A method for obtaining structural information concerning an encoded molecule and method for selecting compounds

Technical Field of the Invention

In one aspect, the present invention relates to a method for obtaining structural information about an encoded molecule. The encoded molecule may be produced by a reaction of a plurality of chemical entities and may be capable of being connected to an identifier oligonucleotide containing codons informative of the identity of the chemical entities which have participated in the formation of the encoded molecule. In a certain embodiment, primers are designed complementary to the codons appearing on the identifier oligonucleotide, and the presence, absence or relative abundance of a codon is evaluated by mixing a primer with the identifier oligonucleotide in the presence of a polymerase and substrate (deoxy)ribonucleotide triphosphates and measuring the extension reaction. In another aspect, the invention provides a method for selecting compounds which binds to a target. More specifically, the invention relates to a method in which a target associated with an oligonucleotide initially is mixed with a library of complexes, each complex comprising a display molecule and an oligonucleotide identifying said display molecule. Next, due an increased proximity, the target oligonucleotide is coupled to the identifier oligonucleotide of complexes having a display molecule with affinity towards the target. In a final stage the coupled nucleotides are analysed to deduce at least the identity of the display molecule.

A method for obtaining structural information concerning an encoded molecule

The below paragraphs up to the section entitled "Method for identifying a display molecule" relate to the first aspect of the invention.

The first aspect of the present invention relates to a method for obtaining structural information about an encoded molecule. The encoded molecule is usually produced by a process that comprises the reaction of a plurality of

chemical entities. The synthesis of the encoded molecule is recorded or programmed in an identifier oligonucleotide which is attached to the encoded molecule. The structural information obtained by the present method may be used to obtain the entire structure of the encoded molecule or a part thereof.

Background of the invention

The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more efficient generation and screening of a huge number of molecules. The approach taken may involve the encoding and/or templating of molecules other than natural biopolymers and a coupling of the molecules to respective templates or identifier parts containing information about the reactants that have participated in the formation of the molecule. These approaches allow the researcher to generate and screen a huge number of molecules at the same time.

In US 5,723,598 it is suggested to prepare libraries of bifunctional molecules, in which one part of the bifunctional molecule comprises an encoded part and the other part of the molecule contains an identifying part. The identifying part is segregated into codons, i.e. a stretches of nucleotides, which codes for reactants that have been involved in the synthesis of the encoded molecule. The libraries of bifunctional molecules are generally prepared by a split-and-mix method, which involves the initial reaction between a nascent bifunctional molecule and a range of different reactants in separate compartments at one end of the nascent bifunctional molecule and a corresponding range of identifier unit oligonucleotides (codons) and the other end. Subsequently, the contents of the compartments are mixed and the mixture is disposed in separate compartments and reacted again with another range of reactants and corresponding codons. Following the generation of a library of the bifunctional molecules, a partitioning with respect to affinity towards a target is conducted and the identifier part of the bifunctional molecule is decoded to establish the chemical structure of the compounds in the library that is likely to be a ligand

to the target. The decoding step implies that the identifier oligonucleotides initially are amplified by PCR. The PCR product is subsequently incorporated in to a suitable vector which is transformed to a host organism, usually E. coli. Following the incubation of the E. coli, colonies are picked and sequenced.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated above. The approach is based on the same split-and-mix strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. A plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the stand a plurality of codon regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to produce a library of typically between 10^3 and 10^6 different compounds. The decoding is performed utilizing the process depicted above.

Recently, a new method for encoding molecules has been suggested, which can be performed in a single "pot". WO 02/00419 and WO 02/103008 disclose methods for preparing virtually any molecule connected to a template coding for chemical entities which have reacted to form the molecule. In short, a template segregated into a plurality of codons and a plurality of building blocks comprising a transferable chemical entity and an anticodon are initially provided. Under hybridisation conditions, the template and building blocks are annealed together and the chemical entities are subsequently reacted to form the molecule. However, after a sufficient number of rounds of selections have been performed, the template must be decoded to establish the identity of the encoded molecule. The decoding step implies that the template oligonucleotides initially are amplified by PCR. The PCR product is

subsequently incorporated in to a suitable vector which is transformed to a host organism, usually E. coli. Following the incubation of the E. coli, colonies are picked and sequenced.

In an aspect of the invention, it is the object to facilitate the decoding of the coding oligonucleotide in order to obtain at least partial structural information of the encoded molecule being a ligand towards a target. In another aspect of the invention, it is desired to obtain information about which chemical entities that result in encoded molecules successful in a selection process. Such chemical entities may be used in the formation of a second generation library.

Summary of the Invention

The first aspect of the present invention concerns a method for obtaining structural information about an encoded molecule produced by a process comprising reaction of a plurality of chemical entities, said encoded molecule being capable of forming part of a complex also comprising an identifier oligonucleotide containing codons informative of the identity of chemical entities which have participated in the formation of the encoded molecule, the method comprises mixing a primer oligonucleotide with the identifier oligonucleotide, subjecting the mixture to a condition allowing for an extension reaction to occur when the primer is sufficient complementary to a part of the identifier oligonucleotide, and evaluating, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons.

The method according to the invention may be performed on a single identifier oligonucleotide or a composition of identifier oligonucleotides to obtain structural information about the encoded molecule or a composition of encoded molecules, respectively, that have been attached to the identifier oligonucleotide(s).

A single identifier may be analysed using the above method to verify the incorporation into the encoded molecule of one or more chemical entities or to deconvolute the identity of the entire encoded molecule. A composition of two or more identifier oligonucleotides generally results from a selection process, i.e. a process involving subjecting a library of different complexes to a condition partitioning the composition from the remainder of the library. Usually the partitioning condition includes an affinity assay in which the library of complexes is contacted with a target and the identifier oligonucleotides of the binding complexes are harvested.

The conditions allowing for an extension reaction to occur may be selected from a enzymatic or chemical means. Suitably, the condition involves one or more enzymes. In a certain embodiment of the invention, the condition which allows for an extension reaction to occur includes a polymerase or a ligase as well as suitable substrates for the enzyme used. Preferred is a polymerase together with a blend of (deoxy)ribonucleotide triphosphates. Suitably, the blend include one or more of dATP, dGTP, dCTP, and dTTP.

A library of complexes can have any appropriate size. Typically, the size is above 10^3, typically above 10^6 different complexes. An effective, extensive, and rapid decoding is therefore desirable. The method of the present invention may be used at various stages of the process of finding a ligand to a certain target. As examples, the method of the invention may be used for controlling the quality of a starting library. The information acquired may be used to verify which codons being present, absent, and, in some embodiments, also the relative abundance. Thus, the method of the invention delivers a reliable picture of the process which has produced the library. If, for some reason, a chemical entity has not been incorporated into the encoded molecules, the absence of a codon for this chemical entity will in certain embodiments of the invention indicate this fact.

Another example of the use of the present method is following the selection. After the selection has been performed the codon profile is indicative of the chemical entities that have been used in the synthesis of encoded molecules having an affinity towards the target. In the event the selection has been sufficiently effective it may be possible directly to deduce a part or the entire structure of binding encoded molecules. Alternatively, it may be possible to deduce a structural unit appearing more frequently among the encoded molecules after the selection, which gives important information to the structure-activity-relationship (SAR). If the selection process has not narrowed the size of the library to a manageable number, the formation of a second generation library may be contemplated. In the formation of the second generation library chemical entities which have not been involved in the synthesis of encoded molecules that have been successful in the selection may be omitted, thus limiting the size of the new library and at the same time increasing the concentration of binding complexes. The second generation library may then be subjected to more stringent selection conditions to allow only the encoded molecules with a higher affinity to bind to the target. The second generation library may also be spiked with certain chemical entities suspected of increasing the performance of the final encoded molecule. The indication of certain successful chemical entities may be obtained from the SAR. The use in a second generation library of chemical entities, which have proved to be interesting for further investigation in a preceding library, may thus entail a shuffling with new chemical entities that may focus the second generation library in a certain desired direction.

The relative abundance of codons may make it possible to decode a plurality of identifiers simultaneously, even in the case when two or more identifiers contain the same codon. Thus, following the formation and selection of a first, second or further generation library, the identity of binding encoded molecules may be partly or entirely deconvoluted by the present method.

In a practical approach the library comprises complexes with identifier oligonucleotides having n codon positions each. In a certain aspect of the invention n is an integer independently selected from of 2 to 8. It may be preferred that n is constant among all the complexes in the library to facilitate the decoding process. Each of the codons in a certain position is in an aspect of the invention selected from a set of m different codons. m may vary for each codon position or may be constant among the various codon positions. It may be preferred in some embodiments to have all the codons in each position selected from the same set of m codons. However, in other embodiments, especially such involving hybridisation in the recognition between the codon and the anticodon, it may be preferred that all the codons are different.

Preferably, any member of the codon set differs from any other codons in the set with the identity of at least one nucleotide, i.e. at least one nucleotide position occurs. In some aspects of the invention it is preferred that any member of the codon set differs with at least two nucleotides nucleotide positions from any other member of the set to increase the fidelity of the method. In general, it is desired to maximize the differences between individual codons of the set. In some embodiments of the invention, a set of primers comprising a sequence of complementing the set of codons are prepared.

In a preferred aspect of the method a framing sequence is related to each of the n codon positions in a particular complex, said framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule. Typically, the framing sequence is identical among the complexes for each of the reaction rounds and is selected from a group of n different nucleotide sequences. In a certain aspect of the invention n x m different primers fully or in part complementing any combination of the set of m different codons and the set of n different framing sequences is prepared. The n x m primers may be used in separate compartments to reveal the identity of a chemical entity as well as the point in time of the synthesis of the encoded molecule is has reacted.

In a particular aspect, the invention concerns a method for identifying the chemical entities utilized in the formation of an encoded molecule or a composition of encoded molecules, wherein in separate compartments, n x m primers individually are mixed with an aliquot of a composition obtained by subjecting a library of different complexes to a condition partitioning said composition from the remainder of the library, subjected to a mixture of polymerase and substrate (deoxy)ribonucleotide triphosphates under conditions allowing for an extension reaction to occur when a primer is sufficient complementary to a part of one or more identifier oligonucleotides present in the aliquot, and evaluation, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons in each compartment.

The invention also concerns a set comprising a collection of oligonucleotide primers, a polymerase, a composition of (deoxy)ribonucleotide triphosphates (dNTPs), and a library of complexes composed of a display molecule part and an identifier oligonucleotide, said oligonucleotide comprising codons informative of the identity of the chemical entities which has participated in the formation of the display molecule, wherein the oligonucleotide primers are sufficiently complementary to codons appearing on the identifier oligo nucleotides in the library to allow for an extension to occur.

Detailed Description of the Invention

Complex

The complex comprises an encoded molecule and an identifier oligonucleotide. The identifier comprises codons that identify the encoded molecule. Preferably, the identifier oligonucleotide identifies the encoded molecule uniquely, i.e. in a library of complexes a particular identifier is capable of distinguishing the molecule it is attached to from the rest of the molecules.

The encoded molecule and the identifier may be attached directly to each other or through a bridging moiety. In one aspect of the invention, the bridging moiety is a selectively cleavable linkage.

The identifier oligonucleotide may comprise two or more codons. In a preferred aspect the identifier oligonucleotide comprises three or more codons. The sequence of each codon can be decoded utilizing the present method to identify reactants used in the formation of the encoded molecule. When the identifier comprises more than one codon, each member of a pool of chemical entities can be identified and the order of codons is informative of the synthesis step each member has been incorporated in.

In a certain embodiment, the same codon is used to code for several different chemical entities. In a subsequent identification step, the structure of the encoded molecule can be deduced taking advantage of the knowledge of different attachment chemistries, steric hindrance, deprotection of orthogonal protection groups, etc. In another embodiment, the same codon is used for a group of chemical entities having a common property, such as a lipophilic nature, a certain attachment chemistry etc. In a preferred embodiment, however, the codon is unique i.e. a similar combination of nucleotides does not appear on the identifier oligonucleotide coding for another chemical entity. In a practical approach, for a specific chemical entity, only a single combination of nucleotides is used. In some aspects of the invention, it may be advantageous to use several codons for the same chemical entity, much in the same way as Nature uses up to six different codons for a single amino acid. The two or more codons identifying the same chemical entity may carry further information related to different reaction conditions.

The sequence of the nucleotides in each codon may have any suitable length. The codon may be a single nucleotide or a plurality of nucleotides. In some aspects of the invention, it is preferred that each codon independently

comprises four or more nucleotides, more preferred 4 to 30 nucleotides. In some aspects of the invention the lengths of the codons vary.

A certain codon may be distinguished from any other codon in the library by only a single nucleotide. However, to facilitate a subsequent decoding process and to increase the ability of the primer to discriminate between codons it is in general desired to have two or more mismatches between a particular codon and any other codon appearing on identifier oligonucleotide. As an example, if a codon length of 5 nucleotides is selected, more than 100 nucleotide combinations exist in which two or more mismatches appear. For a certain number of nucleotides in the codon, it is generally desired to optimize the number of mismatches between a particular codon relative to any other codon appearing in the library.

The identifier oligonucleotide will in general have at least two codons arranged in sequence, i.e. next to each other. Two neighbouring codons may be separated by a framing sequence. Depending on the encoded molecule formed, the identifier may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a suitable framing sequence. Preferably, all or at least a majority of the codons of the identifier are separated from a neighbouring codon by a framing sequence. The framing sequence may have any suitable number of nucleotides, e.g. 1 to 20. Alternatively, codons on the identifier may be designed with overlapping sequences.

The framing sequence, if present, may serve various purposes. In one setup of the invention, the framing sequence identifies the position of the codon. Usually, the framing sequence either upstream or downstream of a codon comprises information which positions the chemical entity and the reaction conditions in the synthesis history of the encodedmolecule. The framing sequence may also or in addition provide for a region of high affinity. The high affinity region may ensure that a hybridisation event with an anti-codon will

occur in frame. Moreover, the framing sequence may adjust the annealing temperature to a desired level.

A framing sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. Examples of nucleobases having this property are guanine and cytosine. Alternatively, or in addition, the framing sequence may be subjected to backbone modification. Several back bone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

The sequence comprising a codon and an adjacent framing sequence has in a certain aspect of the invention a total length of 11 nucleotides or more, preferably 15 nucleotides or more. A primer may be designed to complementary to the codon sequence as well as the framing sequence. The presence of an extension reaction under conditions allowing for such reaction to occur is indicative of the presence of the chemical entity encoded in the codon as well as the position said chemical entity has in the entire synthesis history of the encoded molecule.

The identifier may comprise flanking regions around the coding section. The flanking regions can also serve as priming sites for amplification reactions, such as PCR or as binding region for oligonucleotide probe. The identifier may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block.

It is to be understood that when the term identifier oligonucleotide is used in the present description and claims, the identifier oligonucleotide may be in the sense or the anti-sense format, i.e. the identifier can be a sequence of codons which actually codes for the encoded molecule or can be a sequence

complementary thereto. Moreover, the identifier may be single-stranded or double-stranded, as appropriate.

The encoded molecule part of the complex is generally of a structure expected of having an effect on a target. When the target is of pharmaceutical importance, the encoded molecule is generally a possible drug candidate. The complex may be formed by tagging a library of different possible drug candidates with a tag, e.g. a nucleic acid tag identifying each possible drug candidate. In another embodiment of the invention, the molecule formed by a variety of reactants which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the final molecule displayed on the complex. The post-modification may involve the cleavage of one or more chemical bonds attaching the encoded molecule to the identifier in order more efficiently to display the encoded molecule.

The formation of an encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may react with a reactive group also appearing on the original scaffold or a reactive group incorporated by the first chemical entity. Further chemical entities may be involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule may be mediated by a bridging molecule. As an example, if the nascent encoded molecule and the chemical entity both comprise an amine group a connection between these can be mediated by a dicarboxylic acid. A synthetic molecule is in general produced in vitro and may be a naturally occurring or an artificial substance. Usually, a synthetic molecule is not produced using the naturally translation system in an in vitro process.

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block

prior to the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiments the building blocks also comprise an affinity region providing for affinity towards the nascent complex.

Thus, the chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anti-codon serves the function of transferring the genetic information of the building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order. The chemical entities are preferably reacted without enzymatic interaction in some aspects of the invention. Notably, the reaction of the chemical entities is preferably not mediated by ribosomes or enzymes having similar activity. In other aspects of the invention, enzymes are used to mediate the reaction between a chemical entity and a nascent encoded molecule.

According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on a nucleic acid template. Another method for transferring the genetic information of the anti-codon to the nascent complex is to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the genetic information of the anti-codon to the nascent complex by an extension reaction using a polymerase and a mixture of dNTPs.

The chemical entity of the building block may in most cases be regarded as a precursor for the structural entity eventually incorporated into the encoded molecule. In other cases the chemical entity provides for the eliminations of chemical units of the nascent encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is transferred to a nascent encoded molecule it is to be understood that not necessarily all

the atoms of the original chemical entity is to be found in the eventually formed encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. Non-limiting examples of scaffolds are opiates, steroids, benzodiazepines, hydantoines, and peptidylphosphonates.

The reactive group of the chemical entity may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a chemical reagent or an enzyme. The

cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In some aspects of the invention, it is appropriate to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational spaced sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule.

The encoded molecules may have any chemical structure. In a preferred aspect, the encoded molecule can be any compound that may be

synthesized in a component-by-component fashion. In some aspects the synthetic molecule is a linear or branched polymer. In another aspect the synthetic molecule is a scaffolded molecule. The term "encoded molecule" also comprises naturally occurring molecules like α-polypeptides etc, however produced *in vitro* usually in the absence of enzymes, like ribosomes. In certain aspects, the synthetic molecule of the library is a non-α-polypeptide.

The encoded molecule may have any molecular weight. However, in order to be orally available, it is in this case preferred that the synthetic molecule has a molecular weight less than 2000 Daltons, preferably less than 1000 Dalton, and more preferred less than 500 Daltons.

The size of the library may vary considerably pending on the expected result of the inventive method. In some aspects, it may be sufficient that the library comprises two, three, or four different complexes. However, in most events, more than two different complexes are desired to obtain a higher diversity. In some aspects, the library comprises 1,000 or more different complexes, more preferred 1,000,000 or more different complexes. The upper limit for the size of the library is only restricted by the size of the vessel in which the library is comprised. It may be calculated that a vial may comprise up to 10^{14} different complexes.

Extension reaction

The extension reaction requires a primer, a polymerase as well as a collection of deoxyribonucleotide triphosphates (abbreviated dNTP's herein) to proceed. An extension product may be obtained in the event the primer is sufficient complementary to an identifier oligonucleotide for a polymerase to recognise the double helix as a substrate. After binding of the polymerase to the double helix, the deoxyribonucleotide triphosphates (blend of dATP, dCTP, dGTP, and dTTP) are incorporated into the extension product using the identifier oligonucleotide as template. The conditions allowing for the exten-

sion reaction to occur usually includes a suitable buffer. The buffer may be any aqueous or organic solvent or mixture of solvents in which the polymerase has a sufficient activity. To facilitate the extension process the polymerase and the mixture of dNTP's are generally included in a buffer which is added to the identifier oligonucleotide and primer mixture. An exemplary kit comprising the polymerase and the nNTP's for performing the extension process comprises the following: 50 mM KCl; 10 mM Tris-HCl at pH 8.3; 1.5 mM MgCl2 ; 0.001% (wt/vol) gelatin, 200 µM dATP; 200 µM dTTP; 200 µM dCTP; 200 µM dGTP; and 2.5 units Thermus aquaticus (Taq) DNA polymerase I (U.S. Pat. No. 4,889,818) per 100 microliters (µl) of buffer.

The primer may be selected to be complementary to one or more codons or parts of such codons. The length of the primers may be determined by the length of the codons, however, the primers usually are at least about 11 nucleotides in length, more preferred at least 15 nucleotides in length to allow for an efficient extension by the polymerase. The presence or absence of one or more codons is indicated by the presence of or absence of an extension product. The extension product may be measured by any suitable method, such as size fractioning on an agarose gel and staining with ethidium bromide.

In a preferred embodiment the admixture of identifier oligonucleotide and primer is thermocycled to obtain a sufficient number of copies of the extension product. The thermocycling is typically carried out by repeatedly increasing and decreasing the temperature of the mixture within a temperature range whose lower limit is about 30 degrees Celsius (30°C) to about 55°C and whose upper limit is about 90°C to about 100° C. The increasing and decreasing can be continuous, but is preferably phasic with time periods of relative temperature stability at each of temperatures favouring polynucleotide synthesis, denaturation and hybridization.

When a single complex is analysed in accordance with the present method, the result may be used to verify the presence or absence of a specific chemical entity during the formation of the display molecule. The formation of an extension product is indicative of the presence of an oligonucleotide part complementary to the primer in the identifier oligonucleotide. Conversely, the absence of an extension product is indicative of the absence of an oligonucleotide part complementary to the primer in the identifier oligonucleotide. Selecting the sequence of the primer such that it is complementary to one or more codons will therefore provide information of the structure of the encoded molecule coded for by this codon(s).

In a preferred aspect of the invention, in the mixture of the identifier oligonucleotide and the primer oligonucleotide, a second primer complementary to a sequence of the extension product is included. The second primer is also termed reverse primer and ensures an exponential increase of the number of produced extension products. The method using a forward and reverse primer is well known to skilled person in the art and is generally referred to as polymerase chain reaction (abbreviated PCR) in the present application with claims. In one embodiment of the invention the reverse primer is annealed to a part of the extension product downstream, i.e. near the 3´end of the extension product, or a part complementing the coding part of the identifier oligonucleotide. In another embodiment, the first primer (forward primer) anneals to an upstream position of the identifier oligonucleotide, preferably before the coding part, and the reverse primer anneals to a sequence of the extension product complementing one or more codons or parts thereof.

The amplicons resulting from the PCR process may be stained during or following the reaction to ease the detection. A staining after the PCR process may be prepared with e.g. ethidium bromide or a similar staining agent. As an example, amplicons from the PCR process is run on an agarose gel and subsequently stained with ethidium bromide. Under UV illumination bands of amplicons becomes visible. It is possible to incorporate the staining agent in

the agarose gel or to allow a solution of the staining agent to migrate through the gel. The amplicons may also be stained during the PCR process by an intercalating agent, like CYBR. In presence of the intercalating agent while the amplification proceeds it will incorporate in the double helix. The intercalation agent may then be made visible by irradiation by a suitable source.

The intensity of the staining is informative of the relative abundance of a specific amplicon. Thus, it is possible to quantify the occurrence of a codon in an identifier oligonucleotide. When a library of bifunctional complexes has been subjected to a selection the codons in the pool of identifier oligonucleotides which has been selected can be quantified using this method. As an example a sample of the selected identifier oligonucleotides is subjected to various PCR amplifications with different primers in separate compartments and the PCR product of each compartment is analysed by electrophoresis in the presence of ethidium bromide. The bands that appear can be quantified by a densitometric analysis after irradiation by ultraviolet light and the relative abundance of the codons can be measured.

Alternatively, the primers may be labelled with a suitable small molecule, like biotin or digoxigenin. A PCR-ELISA analysis may subsequently be performed based on the amplicons comprising the small molecule. A preferred method includes the application of a solid support covered with streptavidin or avidin when biotin is used as label and anti-digoxigenin when digoxigenin is used as the label. Once captured, the amplicons can be detected using an enzyme-labelled avidin or anti-dixigenin reporter molecule similar to a standard ELISA format.

To avoid laborious post-PCR handling steps required to evaluate the amplicons, it is in a certain embodiment preferred to measure the extension process "real time". Several real time PCR processes has been developed and all the suitable real time PCR process available to the skilled person in the art can be used in the evaluating step of the present invention and are include in

the present scope of protection. The PCR reactions discussed below are of particular interest.

The monitoring of accumulating amplicons in real time has been made possible by labelling of primers, probes, or amplicons with fluorogenic molecules. The real time PCR amplification is usually performed with a speed faster than the conventional PCR, mainly due to reduced cycles time and the use of sensitive methods for detection of emissions from the fluorogenic labels. The most commonly used fluorogenic oligoprobes rely upon fluorescent resonance energy transfer (FRET) between fluorogenic labels or between one flourophor and a dark or "black-hole" non-fluorescent quencher (NFQ), which disperse energy as heat rather than fluorescence. FRET is a spectroscopic process by which energy is passed between molecules separated by 10-100 Å that have overlapping emission and absorption spectra. An advantage of many real time PCR methods is that they can be carried out in a closed system, i.e. a system which does not need to be opened to examine the result of the PCR. A closed system implies a reduced result turnaround, minimisation of the potential for carry-over contamination and the ability to closely scrutinise the essay's performance.

The present real time PCR methods currently available to the skilled person can be classified into either amplicon sequence specific or non-specific methods. The basis for the non-specific detection methods is a DNA-binding fluorogenic molecule. Included in this class are the earliest and simplest approaches to real time PCR. Ethidium bromide, YO-PRO-1, and SYBR® green 1 all fluorescence when associated with double stranded DNA which is exposed to a suitable wavelength of light. This approach requires the fluorescent agent to be present during the PCR process and provides for a real time detection of the fluorescent agent as it is incorporated into the double stranded helix.

The amplicons sequence specific methods includes, but are not limited to, the TaqMan®, hairpin, LightCycler®, Sunrise®, and Scorpion® methods. The LightCycler® method also designated "HybProbes" make use of a pair of adjacent, fluorogenic hybridisation oligonucleotide probes. A first, usually the upstream oligoprobe is labelled with a 3' donor fluorophore and the second, usually the downstream probe is commonly labelled with either a Light cycler Red 640 or Red 705 acceptor fluorophore a the 5' terminus so that when both oligoprobes are hybridised the two fluorophores are located in close proximity, such as within 10 nm, of each other. The close proximity provides for the emission of a fluorescence when irradiated with a suitable light source, such a blue diode in case of the LightCycler®. The region for annealing of the probes may be any suitable position that does not interfere with the primer annealing. In a suitable setup, the site for binding the probes are positioned downstream of the codon region on the identifier oligonucleotide. Alternatively, when a reverse primer is used, the region for annealing the probes may be at the 3' end of the strand complementing the identifier oligonucleotide. Another embodiment of the LightCycler method includes that the pair of oligonucleotide probes are annealed to one or more codons and primer sites exterior to the coding part of the identifier oligonucleotide are used for PCR amplification.

The TaqMan® method, also referred to as the 5' nuclease or hydrolysis method, requires an oligoprobe, which is attached to a reporter flourophor, such as 6-carboxy-fluoroscein, and a quencher fluorophore, such as 6-carboxy-tetramethyl-rhodamine, at each end. When in close proximity, i.e. annealed to an identifier oligonucleotide, or a sequence complementing the identifier oligonucleotide, the quencher will "hijack" the emissions that have resulted from the excitation of the reporter. As the polymerase progresses along the relevant strand, it displaces and the hydrolyses the oligoprobe via its 5'→3' endonuclease activity. Once the reporter is removed from the extinguishing influence of the quencher, it is able to release excitation energy at a wavelength that can be monitored by a suitable instrument, such as ABI

Prism® 7700. The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise is normally used as an indicator of successful identifier oligonucleotide amplification. This threshold cycle (C_T) is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicons exceeds 10 standard deviations of the mean base line fluorescence. The C_T is proportional to the number of identifier oligonucleotide copies present in the sample. The TaqMan probe is usually designed to hybridise at a position downstream of a primer binding site, be it a forward or a reverse primer. When the primer is designed to anneal to one or more codons of the identifier oligonucleotide, the presence of these one or more codons is indicated by the emittance of light. Furthermore, the quantity of the identifier oligonucleotides comprising the one or more codons may be measured by the C_T value.

The Hairpin method involves an oligoprobe, in which a fluorophore and a quencher are positioned at the termini. The labels are hold in close proximity by distal stem regions of homologous base pairing deliberately designed to create a hairpin structure which result in quenching either by FRET or a direct energy transfer by a collisional mechanism due to the intimate proximity of the labels. When direct energy transfer by a collision mechanism is used the quencher is usually different from the FRET mechanism, and is suitably 4-(4'-dimethylamino-phenylazo)-benzene (DABCYL). In the presence of a complementary sequence, usually downstream of a primer, or within the bounds of the primer binding sides in case of more than one a single primer, the oligoprobe will hybridise, shifting into an open configuration. The fluorophore is now spatially removed from the quencher's influence and fluorescence emissions are monitored during each cycle. In a certain aspect, the hairpin probe may be designed to anneal to a codon in order to detect this codon if present on the identifier oligonucleotide. This embodiment may be suitable if codons only differs from each other with a single or a few nucleotides, because is in well-known that the occurrence of a mismatch between a hairpin oligoprobe and its target sequence has a greater destabilising effect

on the duplex than the introduction of an equivalent mismatch between the target oligonucleotide and a linear oligoprobe. This is probably because the hairpin structure provides a highly stable alternate conformation.

The Sunrise and Scorpion methods are similar in concept to the hairpin oligoprobe, except that the label becomes irreversible incorporated in to the PCR product. The Sunrise method involves a primer (commercially available as Amplifluor™ hairpin primers) comprising a 5' fluorophore and a quencher, e.g. DABCYL. The labels are separated by complementary stretches of sequence that create a stem when the sunrise primer is closed. At the 3' terminus is a target specific primer sequence. In a preferred embodiment the target sequence is a codon, optionally more codons. The sunrise primer's sequence is intended to be duplicated by the nascent complementary stand and, in this way, the stem is destabilised, the two fluorophores are held apart, usually between 15 and 25 nucleotides, and the fluorophore is free to emit its excitation energy for monitoring. The Scorpion primer resembles the sunrise primer, but derivate in having a moiety that blocks duplication on the signalling portion of the scorpion primer. The blocking moiety is typically hexethylene glycol. In addition to the difference in structure, the function of the scorpion primers differs slightly in that the 5' region of the oligonucleotide is designed to hybridise to a complementary region within the amplicons. In a certain embodiment the complementary region is a codon on the identifier oligonucleotide. The hybridisation forces the labels apart disrupting the hairpin and permitting emission in the same way as the hairpin probes.

Methods for forming a library of complexes

The complexes comprising an identifier part having two or more codons that code for reactants that have reacted in the formation of the encoded molecule part of the complex may be formed by a variety of processes. Generally, the preferred methods can be used for the formation of virtually any kind of encode molecule. Suitable examples of processes include prior art methods disclosed in WO 93/20242, WO 93/06121, WO 00/23458, WO 02/074929,

and WO 02/103008, the content of which being incorporated herein by reference as well as methods of the present applicant not yet public available, including the methods disclosed in DK PA 2002 01955 filed 19 December 2002, and DK PA 2003 00430 filed 20 March 2003. Any of these methods may be used, and the entire content of the patent applications are included herein by reference.

Below four preferred embodiments are described. A first embodiment disclosed in more detail in WO 02/103008 is based on the use of a polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides is provided. Subsequently primers are annealed to each of the templates and a polymerase is extending the primer using nucleotide derivatives which have appended chemical entities. Subsequent to or simultaneously with the incorporation of the nucleotide derivatives, the chemical entities are reacted to form a reaction product. The encoded molecule may be post-modified by cleaving some of the linking moieties to better present the encoded molecule.

Several possible reaction approaches for the chemical entities are apparent. First, the nucleotide derivatives can be incorporated and the chemical entities subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide bond. Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such as an ester or a thioester group. An adjacent building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

A second embodiment for obtainment of complexes pertains to the use of hybridisation of building blocks to an identifier oligonucleotide and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed such that they recognise a sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codon and the codon to each other a reaction of the chemical entity is effected.

The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

A third embodiment for the generation of a complex includes chemical or enzymatical ligation of building blocks when these are lined up on a template. Initially, templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product. The method is disclosed in more detail in DK PA 2003 00430 filed 20 March 2003.

A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region is annealed to a building block comprising a region complementary to the affinity section. Subsequently the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may

be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon. This method is disclosed in detail in DK PA 2002 01955 filed 19 December 2002.

After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however at least one linker must be maintained to provide for the complex.

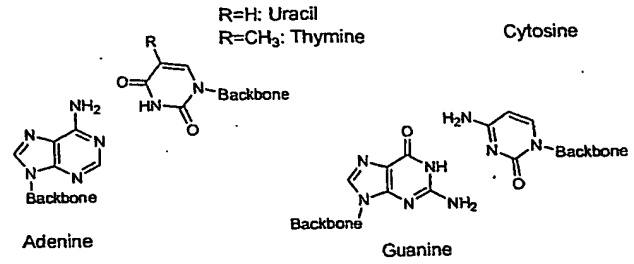
Nucleotides

The nucleotides used in the present invention may be linked together in a sequence of nucleotides, i.e. an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a back-bone. The back bone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

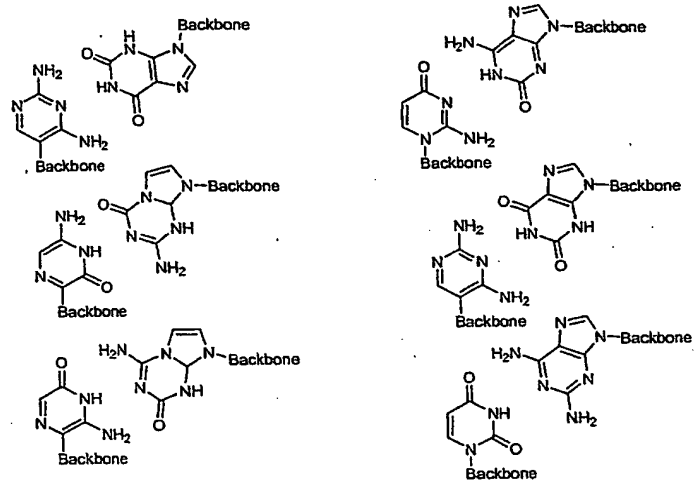
The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosin, N^6,N^6-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C^3-C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

Examples of suitable specific pairs of nucleobases are shown below:

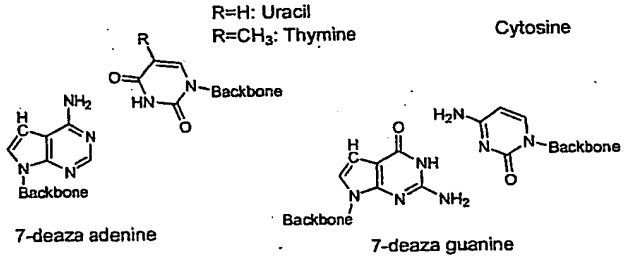
Natural Base Pairs



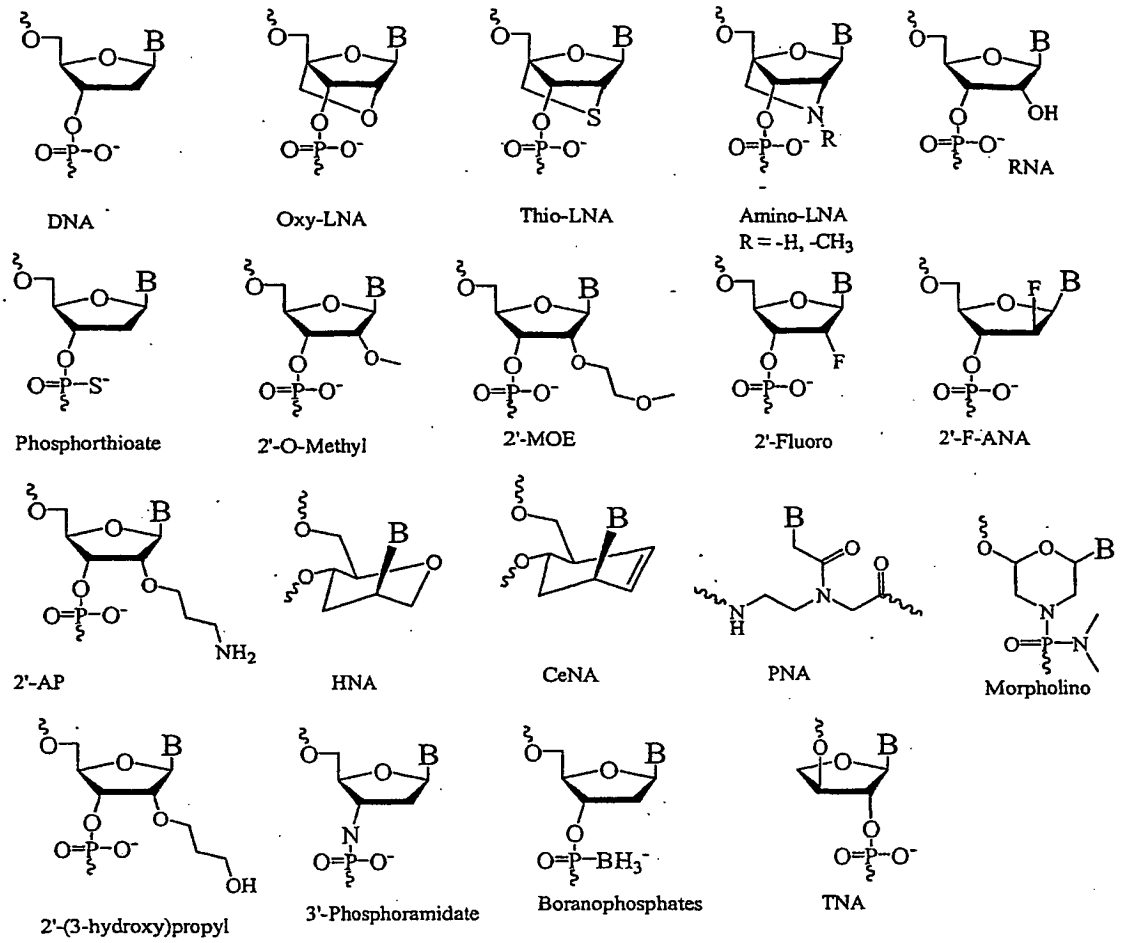
Synthetic Base Pairs



Synthetic purine bases pairing with natural pyrimidines



Suitable examples of backbone units are shown below (B denotes a nucleo-
base):



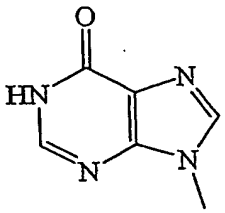
The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-flour-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2-deoxyribose. The internucleoside linkage may be the natural occurring phospodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the

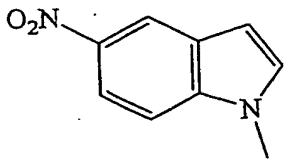
internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as universal base because inosine can pair nearly isoenergetically with A, T, and C. Other compounds having the same ability of non-specifically base-pairing with natural nucleobases have been formed. Suitable compounds which may be utilized in the present invention includes among others the compounds depicted below

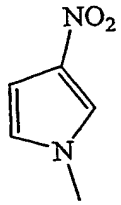
Examples of Universal Bases:



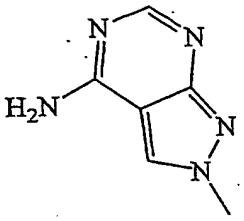
Inosine



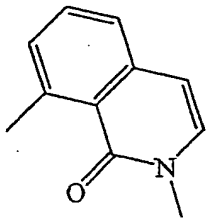
5-Nitroindole



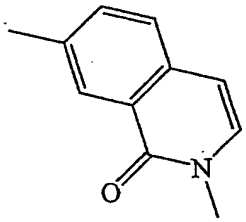
3-Nitropyrrole



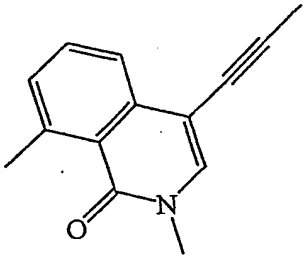
N^8-8aza-7deazaadenine



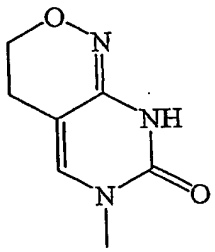
MICS



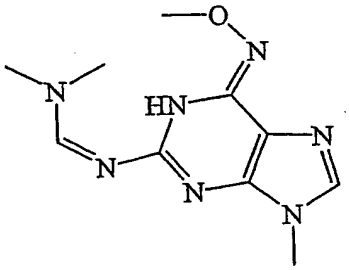
5MICS



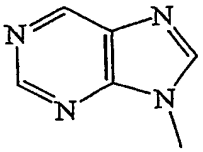
PIM



dP



dK



Nebularine

Building block

The chemical entities that are precursors for structural additions or elimina-

5 tions of the encoded molecule may be attached to a building block prior to

the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly

or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule or reactive site.

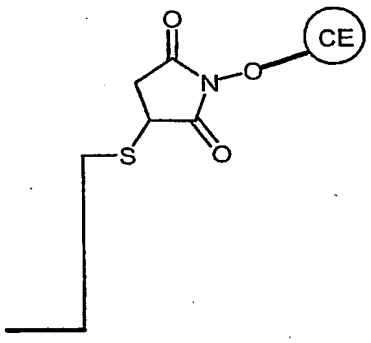
The anticodon complements the codon of the identifier sequence and generally comprises the same number of nucleotides as the codon. The anticodon may be adjoined with a fixed sequence, such as a sequence complementing a framing sequence.

Various specific building blocks are envisaged. Building blocks of particular interest are shown below.

Specific Building blocks

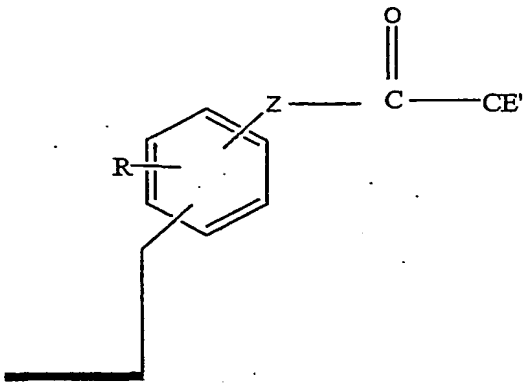
Building blocks transferring a chemical entity to a recipient nucleophilic group

The building block indicated below is capable of transferring a chemical entity (CE) to a recipient nucleophilic group, typically an amine group. The bold lower horizontal line illustrates the building block and the vertical line illustrates a spacer. The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold



The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold, to transfer the chemical entity to the scaffold, thus converting the remainder of the fragment into a leaving group of the reaction. When the chemical entity is connected to the activator through an carbonyl group and the recipient group is an amine, the bond formed on the scaffold will an amide bond. The above building block is the subject of the Danish patent application No. PA 2002 01946 and the US provisional patent application No. 60/434,439, the content of which are incorporated herein in their entirety by reference.

Another building block which may form an amide bond is

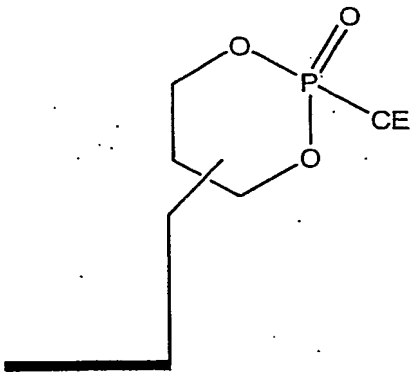


R may be absent or NO_2, CF_3, halogen, preferably Cl, Br, or I, and Z may be S or O. This type of building block is disclosed in Danish patent application No. PA 2002 0951 and US provisional patent application filed 20 December 2002 with the title "A building block capable of transferring a functional entity to a recipient reactive group". The content of both patent application are incorporated herein in their entirety by reference.

A nucleophilic group can cleave the linkage between Z and the carbonyl group thereby transferring the chemical entity –(C=O)-CE' to said nucleophilic group.

Building blocks transferring a chemical entity to a recipient reactive group forming a C=C bond

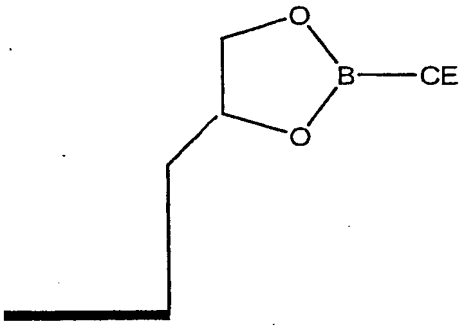
A building block as shown below are able to transfer the chemical entity to a recipient aldehylde group thereby forming a double bond between the carbon of the aldehyde and the chemical entity



The above building block is comprised by the Danish patent application No. DK PA 2002 01952 and the US provisional patent application filed 20 December 2002 with the title "A building block capable of transferring a functional entity to a recipient reactive group forming a C=C double bond". The content of both patent applications are incorporated herein in their entirety by reference.

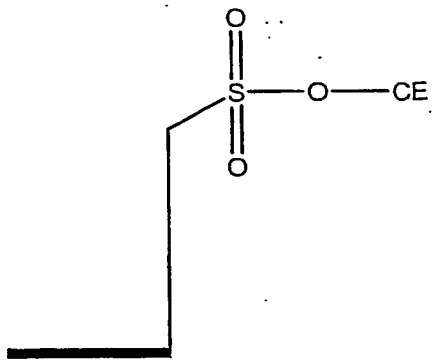
Building blocks transferring a chemical entity to a recipient reactive group forming a C-C bond

The below building block is able to transfer the chemical entity to a recipient group thereby forming a single bond between the receiving moiety, e.g. a scaffold, and the chemical entity.



The above building block is comprised by the Danish patent application No. DK PA 2002 01947 and the US provisional patent application No 60/434,428. The content of both patent applications are incorporated herein in their entirety by reference.

Another building block capable of transferring a chemical entity to a receiving reactive group forming a single bond is



The receiving group may be a nucleophile, such as a group comprising a hetero atom, thereby forming a single bond between the chemical entity and the hetero atom, or the receiving group may be an electronegative carbon atom, thereby forming a C-C bond between the chemical entity and the scaffold.

The chemical entity attached to any of the above building blocks may be a selected from a large arsenal of chemical structures. Examples of chemical entities are

H or entities selected among the group consisting of a C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9.

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, -CNO, $-C(NOH)NH_2$, -NHOH, $-NHNHR^6$, $-C(O)R^6$, $-SnR^6_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of

C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

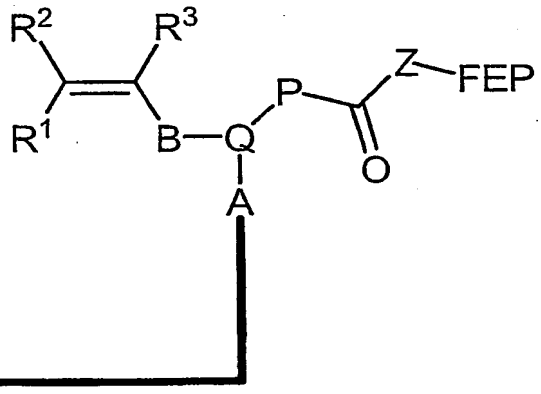
where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and R^7 is independently selected from $-NO_2$, $-COOR^6$, $-COR^6$, -CN, $-OSiR^6_3$, $-OR^6$ and $-NR^6_2$.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, $-NO_2$, $-R^3$, $-OR^3$, $-SiR^3_3$

R^9 is =O, -F, -Cl, -Br, -I, -CN, $-NO_2$, $-OR^6$, $-NR^6_2$, $-NR^6$-$C(O)R^8$, $-NR^6$-$C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6_2$ and $-S(O)_2NR^6_2$.

Cross-link cleavage building blocks

It may be advantageous to split the transfer of a chemical entity to a recipient reactive group into two separate steps, namely a cross-linking step and a cleavage step because each step can be optimized. A suitable building block for this two step process is illustrated below:



Initally, a reactive group appearing on the functional entity precursor (abbreviated FEP) reacts with a recipient reactive group, e.g. a reactive group appearing on a scaffold, thereby forming a cross-link. Subsequently, a cleavage is performed, usually by adding an aqueous oxidising agent such as I_2, Br_2,

Cl_2, H^+, or a Lewis acid. The cleavage results in a transfer of the group HZ-FEP- to the recipient moiety, such as a scaffold.

In the above formula

Z is O, S, NR^4

Q is N, CR^1

P is a valence bond, O, S, NR^4, or a group C_{5-7}arylene, C_{1-6}alkylene, C_{1-6}O-alkylene, C_{1-6}S-alkylene, NR^1-alkylene, C_{1-6}alkylene-O, C_{1-6}alkylene-S option said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

B is a group comprising D-E-F, in which

D is a valence bond or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

E is, when present, a valence bond, O, S, NR^4, or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

F is, when present, a valence bond, O, S, or NR^4,

A is a spacing group distancing the chemical structure from the complementing element, which may be a nucleic acid,

R^1, R^2, and R^3 are independent of each other selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

FEP is a group selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3

R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, -CNO, $-C(NOH)NH_2$, -NHOH, $-NHNHR^6$, $-C(O)R^6$, $-SnR^6_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and R^7 is independently selected from $–NO_2$, $-COOR^6$, $-COR^6$, -CN, $-OSiR^6_3$, $-OR^6$ and $-NR^6_2$.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, $–NO_2$, $-R^3$, $-OR^3$, $-SiR^3_3$

R^9 is =O, -F, -Cl, -Br, -I, -CN, $-NO_2$, $-OR^6$, $-NR^6_2$, $-NR^6$-$C(O)R^8$, $-NR^6$-$C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6_2$ and $-S(O)_2NR^6_2$.

In a preferred embodiment Z is O or S, P is a valence bond, Q is CH, B is CH_2, and R^1, R^2, and R^3 is H. The bond between the carbonyl group and Z is cleavable with aqueous I_2.

Partitioning

The partitioning step, by which the library of bifunctional molecules is subjected to a condition partitioning one or more complexes having a certain property from the remainder of the library, may be referred to as the enrichment step or the selection step, as appropriate, and includes the screening of the library for encoded molecules having predetermined desirable characteristics. Predetermined desirable characteristics can include binding to a target,

catalytically changing the target, chemically reacting with a target in a manner which alters/modifies the target or the functional activity of the target, and covalently attaching to the target as in a suicide inhibitor.

In theory, molecules of interest can be selected based on their properties using either physical or physiological procedures. The method preferred according to the present invention is to enrich molecules with respect to binding affinity towards a target of interest. In a certain embodiment, the basic steps involve mixing the library of complexes with the immobilized target of interest. The target can be attached to a column matrix or microtitre wells with direct immobilization or by means of antibody binding or other high-affinity interactions. In another embodiment, the target and displayed molecules interact without immobilisation of the target. Displayed molecules that bind to the target will be retained by a filter, size-exclusion chromatography *etc*, while non-binding displayed molecules will be removed during a single or a series of wash steps. The identifiers of complexes bound to the target can then be separated by cleaving a physical connection to the encoded molecule or the entire complex may be eluted. It may be considered advantageously to perform a chromatography step after or instead of the washing step. After the cleavage of the physical link between the synthetic molecule and the identifier, the identifier may be recovered from the media and optionally amplified before the decoding step.

A significant reduction in background binders may be obtained with increased washing volumes, repeating washing steps, higher detergent concentrations and prolonged incubation during washing. Thus, the more volume and number of steps used in the washing procedure together with more stringent conditions will more efficiently remove non-binders and background binders. The right stringency in the washing step can also be used to remove low-affinity specific binders. However, the washing step will also remove wanted binders if too harsh conditions are used.

A blocking step, such as incubation of solid phase with skimmed milk proteins or other inert proteins and/or mild detergent such as Tween-20 and Triton X-100, may also be used to reduce the background. The washing conditions should be as stringent as possible to remove background binding but to retain specific binders that interact with the immobilized target. Generally, washing conditions are adjusted to maintain the desired affinity binders, e.g. binders in the micromolar, nanomolar, or pocomolar range.

The target can be any compound of interest. E.g. the target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analogue, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc. without limitation. Suitable targets include, but are not limited to, angiotensin converting enzyme, renin, cyclooxygenase, 5-lipoxygenase, IIL- 1 0 converting enzyme, cytokine receptors, PDGF receptor, type II inosine monophosphate dehydrogenase, β-lactamases, integrin, proteases like factor VIIa, kinases like Bcr-Abl/Her, phosphotases like PTP-1B, and fungal cytochrome P-450. Targets can include, but are not limited to, bradykinin, neutrophil elastase, the HIV proteins, including *tat, rev, gag, int,* RT, nucleocapsid etc., VEGF, bFGF, TGFβ, KGF, PDGF, GPCR, thrombin, substance P, IgE, sPLA2, red blood cells, glioblastomas, fibrin clots, PBMCs, hCG, lectins, selectins, cytokines, ICP4, complement proteins, etc.

A target can also be a surface of a non-biological origin, such as a polymer surface or a metal surface. The method of the invention may then be used to identify suitable coatings for such surfaces.

In a preferred embodiment, the desirable synthetic molecule acts on the target without any interaction between the nucleic acid attached to the desirable encoded molecule and the target. In one embodiment, the bound complex-target aggregate can be partitioned from unbound complexes by a number of methods. The methods include nitrocellulose filter binding, column chroma-

tography, filtration, affinity chromatography, centrifugation, and other well known methods. A preferred method is size-exclusion chromatography.

Briefly, the library of complexes is subjected to the target, which may include contact between the library and a column onto which the target is immobilised. Identifiers associated with undesirable encoded molecules, i.e. synthetic molecules not bound to the target under the stringency conditions used, will pass through the column. Additional undesirable encoded molecules (e.g. encoded molecules which cross-react with other targets) may be removed by counter-selection methods. Desirable complexes are bound to the column. The target may be immobilized in a number of ways. In one embodiment, the target is immobilized through a cleavable physical link, such as one more chemical bonds. The aggregate of the target and the complex may then be subjected to a size exclusion chromatography to separate the aggregate from the rest of the compounds in the media. The complex may then be eluted from the target by changing the conditions (e.g., salt, pH, surfactant, temperature etc.). Alternatively, the complex may be provided with a cleavable linker, preferable orthogonal to a cleavable linker that attaches the target to the solid support, at a position between the synthetic molecule and the identifier. Subsequent to the size exclusion chromatography this cleavable linker is cleaved to separate the identifiers of complexes having affinity towards the targets. Just to mention a single type of orthogonal cleavable linkages, one could attach the target to the solid support through a linkage that can be cleaved by a chemical agent, and the linker separating the synthetic molecule and the identifier may be selected as a photocleavable linkage. More specifically, the former linkage may be a disulphide bond that can be cleaved by a suitable reducing agent like DTT (dithiothreitol) and the latter linkage may be a *o*-nitrophenyl group.

There are other partitioning and screening processes which are compatible with this invention that are known to one of ordinary skill in the art. In one embodiment, the products can be fractionated by a number of common

methods and then each fraction is assayed for activity. The fractionization methods can include size, pH, hydrophobicity, etc.

Inherent in the present method is the selection of encoded molecules on the basis of a desired function; this can be extended to the selection of molecules with a desired function and specificity. Specificity can be required during the selection process by first extracting complexes which are capable of interacting with a non-desired "target" (negative selection, or counter-selection), followed by positive selection with the desired target. As an example, inhibitors of fungal cytochrome P-450 (fungicides) are known to cross-react to some extent with mammalian cytochrome P-450 (resulting in serious side effects). Highly specific inhibitors of the fungal cytochrome could be selected from a library by first removing those complexes capable of interacting with the mammalian cytochrome, followed by retention of the remaining products which are capable of interacting with the fungal cytochrome.

Brief Description of the Figures

Fig. 11 discloses two embodiments of using a Taqman probe (5' nuclease probe) in the measurement of the presence or absence of a certain codon.

Fig. 12 discloses a standard curve used in example 3.

Fig. 13 discloses the result of two experiments reported in example 3.

Example 3 – illustrating the first aspect of the present invention

A preferred embodiment of the invention utilizing a universal Taqman probe is shown in Fig.11. Four codons are shown (P1 through P4; bold pattern) along with flanking regions (light pattern). A universal Taqman probe anneals to a region adjacent to the codon region, but within the amplicon defined by the universal PCR primers Pr.1 and Pr. 2. These primers could be the same as used for amplification of the identifier oligonucleotides encoding binders after an enrichment process on a specific target. However, if minimal length templates are preferred during the encoding process, the region involved in Taqman probe annealing could be appended to the library identifier oligonu-

cleotides by e.g. overlap PCR, ligation, or by employing a long downstream PCR primer containing the necessary sequences. The added length corresponding to the region necessary for annealing of the Taqman probe would be form 20 to 40 nts depending on the type of TaqMan probe and T_A of the PCR primers. The Q-PCR reactions are preferably performed in a 96- or 384-well format on a real-time PCR thermocycling machine.

Fig. 11A shows the detection of abundance of a specific codon sequence in position one. Similar primers are prepared for all codon sequences. For each codon sequence utilized to encode a specific BB in the library a Q-PCR reaction is performed with a primer oligonucleotide complementary to the codon sequence in question. A downstream universal reverse primer Pr. 2 is provided after the Taqman probe to provide for an exponential amplification of the PCR amplicon. The setup is most suited for cases where the codon constitutes a length corresponding to a length suitable for a PCR primer.

Fig. 11B shows the detection of abundance of a specific codon sequence in a specific codon position using a primer which is complementing a codon and a framing sequence. Similar primers are used for all the codons and framing sequences. For each codon sequence utilized to encode a specific BB at a specific codon position in the library a Q-PCR reaction is performed with an oligo complementary to the codon sequence in question as well as a short region up- or downstream of the codon region which ensures extension of the primer in a PCR reaction only when annealed to the codon sequence in that specific codon position. The number of specific primers and Q-PCR reactions needed to cover all codon sequences in all possible codon positions equals the number of codon sequences times the number of codon positions. Thus, monitoring the abundance of 96 different codon sequences in 4 different positions can be performed in a single run on four 96 wells micro titre plates (as shown in Fig. 11B) or a single 384 well plate on a suitable instrument. This architecture allows for the decoding of a $8{,}5 \times 10^7$ library of different encoded molecules.

Quantification is performed relative to the amount of full-length PCR product obtained in a parallel control reaction on the same input material performed with the two external PCR primers Pr.1 + Pr. 2. Theoretically, a similar rate of accumulation of this control amplicon compared to the accumulation of a product utilizing a single codon + sequence specific primer would indicate a 100% dominance of this particular sequence in the position in question.

Although the setups shown in Fig. 11A and 11B employ a Taqman probe strategy, other detection systems (SYBR green, Molecular Beacons etc.) could be utilized. In theory, multiplex reactions employing up to 4 different fluorofors in the same reaction could increase throughput correspondingly.

An example of how a deconvolution process of a library of encoded molecules occurs is described in the following. Imagine that at the end of a selection scheme a pool of 3 ligand families (and the corresponding coding templates) are dominating the population and present at approx. the same concentration. Three different chemical entities are present in the first position of the encoded compounds, and each of these chemical entities are present in combination with one unique chemical entity out of 3 different chemical entities in position P2. Only one chemical entity in position 3 gives rise to active binders, whereas any of a 20% subset of chemical entities (*e.g.* determined by charge, size or other characteristica) are present in position 4. The outcome of the initial codon profile analysis would be: 3 codon sequences are equally dominating in position P1, 3 other codon sequences in position P2, 1 unique codon sequence is dominant in P3 whereas somewhat similarly increased levels of 20% of the codon sequences (background levels of the remaining 80% sequences) are seen in P4. In such cases it could be relevant to use an iterative Q-PCR ("IQ-PCR") strategy to perform a further deconvolution of a library after selection. Again with reference to the example above, by taking the PCR products from the 3 individual wells that contained primers giving the high yields in position P1, diluting the product appropriately and

performing a second round of Q-PCR on each of these identifier oligonucleotides separately, it would be possible to deduce which codon sequence(s) is preferred in P2 when a given codon sequence is present in P1.

Experimental example: The 10 templates used for Q-PCR quantification

Taqman MGB probe binding region: *=AATTCCAGCTTCTAGGAAGAC

P1 P2 P3 P4

5'-CAGCTTGGACACCACGTCATACTAGCTGCTAGAGATGTGGTGATATTAGTGTGTGACGATGGTACGCACAAGTACGAACGTGCATCAGAGAGGACGAGCAGGACCTGGAACCTGGTGC*TTCCTCCACCACGTCTCTGAC-3'

GGAAGAAGACAGAAGACCTG	CTCGACCACTGCAGGTGGAGCTCC
TCAGGAGTCGAGAACTGAAG	CGTGCTTCCTCTGCTGCACCACCG
TGTGTACGTCAACACGTCAG	CCTGGTGTCGAGGTGAGCAGCAGC
TGTGGAACTACCATCCAAGG	CTCGACGAGGTCCATCCTGGTCGC
CCATCCAACATCGTTGGAAG	CGTGAGGAGCAGGTCCTCCTGTCG
AACCTGTCCTGTGAGATCTG	CCTGACACTGGTCGTGGTCGAGGC
TCACGAAGCTGGATGATGAG	CCATCTCGACGACCTGCTCCTGGG
TAGCATCGATCGAACGTAGG	CCACGAGGTCTCCACTGGTCCAGG
TCGAAGCTACTGTCGAGATG	CCACTGAGCTGCTCCTCCAGGTGG

Oligos for template synthesis:

FPv2: CAGCTTGGACACCACGTCATAC
RPv2: GTCAGAGACGTGGTGGAGGAA

Temp1-1: CAGCTTGGACACCACGTCATACTAGCTGCTAGAGATGTGGTGATATTAGTGTGTGACGAT
Temp1-2: CAGCTTGGACACCACGTCATACGGAAGAAGACAGAAGACCTGATATTAGTGTGTGACGAT
Temp1-3: CAGCTTGGACACCACGTCATACTCAGGAGTCGAGAACTGAAGATATTAGTGTGTGACGAT
Temp1-4: CAGCTTGGACACCACGTCATACTGTGTACGTCAACACGTCAGATATTAGTGTGTGACGAT
Temp1-5: CAGCTTGGACACCACGTCATACTGTGGAACTACCATCCAAGGATATTAGTGTGTGACGAT
Temp1-6: CAGCTTGGACACCACGTCATACCCATCCAACATCGTTGGAAGATATTAGTGTGTGACGAT
Temp1-7: CAGCTTGGACACCACGTCATACAACCTGTCCTGTGAGATCTGATATTAGTGTGTGACGAT
Temp1-8: CAGCTTGGACACCACGTCATACTCACGAAGCTGGATGATGAGATATTAGTGTGTGACGAT
Temp1-9: CAGCTTGGACACCACGTCATACTAGCATCGATCGAACGTAGGATATTAGTGTGTGACGAT
Temp1-10:CAGCTTGGACACCACGTCATACTCGAAGCTACTGTCGAGATGATATTAGTGTGTGACGAT

Temp2: GTCCTCTCTGATGCACGTTCGTACTTGTGCGTACCATCGTCACACACTAATATC

Temp3-1: GAACGTGCATCAGAGAGGACGAGCAGGACCTGGAACCTGGTGCAATTCCAGCTTCTAGGAAGACT
Temp3-2: GAACGTGCATCAGAGAGGACTCGACCACTGCAGGTGGAGCTCCAATTCCAGCTTCTAGGAAGACT
Temp3-3: GAACGTGCATCAGAGAGGACGTGCTTCCTCTGCTGCACCACCGAATTCCAGCTTCTAGGAAGACT
Temp3-4: GAACGTGCATCAGAGAGGACCTGGTGTCGAGGTGAGCAGCAGCAATTCCAGCTTCTAGGAAGACT
Temp3-5: GAACGTGCATCAGAGAGGACTCGACGAGGTCCATCCTGGTCGCAATTCCAGCTTCTAGGAAGACT
Temp3-6: GAACGTGCATCAGAGAGGACGTGAGGAGCAGGTCCTCCTGTCGAATTCCAGCTTCTAGGAAGACT
Temp3-7: GAACGTGCATCAGAGAGGACCTGACACTGGTCGTGGTCGAGGCAATTCCAGCTTCTAGGAAGACT
Temp3-8: GAACGTGCATCAGAGAGGACCATCTCGACGACCTGCTCCTGGGAATTCCAGCTTCTAGGAAGACT
Temp3-9: GAACGTGCATCAGAGAGGACCACGAGGTCTCCACTGGTCCAGGAATTCCAGCTTCTAGGAAGACT
Temp3-10:GAACGTGCATCAGAGAGGACCACTGAGCTGCTCCTCCAGGTGGAATTCCAGCTTCTAGGAAGACT

Temp4: GTCAGAGACGTGGTGGAGGAAGTCTTCCTAGAAGCTGGAATT

Synthesis of identifier oligonucleotides:

The 10 identifier oligonucleotides were assembled in 10 seperate 50 µl PCR reactions each containing 0.05 pmol of the oligos Q-Temp1-X, Q-Temp2, Q-Temp3-X and Q-Temp4 (x=1 through 10) and 25 pmol of the external primers FPv2 and RPv2 with TA= 53°C. The 160 bp products were gel-purified using QIAquick Gel Extraction Kit from QIAGEN (Cat. No. 28706) and quantified on spectrophotometer. As a control, 20 ng of each of the templates (as estimated from these measurements) were loaded on an agarose gel.

Preparation of samples for Q-PCR:

Sample A: Generated by mixing 20 ng from each identifier oligonucleotide prep. Volume was adjusted to 50 µl. Concentration: 4 ng/µl = 38.46 fmol/µl (160bp x 650 Da/bp =1.04x105 g/mol. 1 ng= 9.615 fmol). Diluted to 10^7 copies/5µl (0.00332 fmol/µl).

Sample B: 20 ng/20µl stocks of each template were prepared. The sample was mixed as follows:

5µl undil. Template #10
5µl 2x dil. Template #9
5µl 4x dil. Template #8
5µl 8x dil. Template #7
5µl 16x dil. Template #6
5µl 32x dil. Template #5
5µl 64x dil. Template #4
5µl 128x dil. Template #3
5µl 256x dil. Template #2
5µl 512x dil. Template #1

Concentration: 10ng/50µl= 0.20 ng/µl = 1.923 fmol/µl. Diluted 579.2-fold to 10^7 copies/5µl (0.00332 fmol/µl).

Standard curve: The samples for the standard curve was prepared by diluting Sample A 116.55-fold to 10^9 copies/5 µl (0.33 fmol//µl) and subsequently per-

forming a 10-fold serial dilution of this sample. 5 µl was used for each PCR reaction. The standard curve is shown in Fig. 12.

Q-PCR reactions

For 5 ml premix (for one 96-well plate):
2.5 ml Taqman Universal PCR Master Mix (Applied Biosystems; includes Taq polymerase, dNTPs and optimized Taq pol. buffer)
450 µl RPv2 (10 pmol/ul)
25 µl Taqman probe (6-FAM-TCCAGCTTCTAGGAAGAC-MGBNFQ; 50 µM; Applied Biosystems)
1075 µl H2O

40.5 µl premix was aliquoted into each well and 4.5 µl of relevant upstream PCR primer (FPv2 (for standard curve) or one of the codon specific primers listed below; 10 pmol/µl) and 5 µl sample (H2O in wells for negative controls) was added. The codon-specific PCR primers were: (Tm calculations shown are from Vector NTI; matched to Tm for RPv2 (67.7°C))

P1-1:	GTCATACTAGCTGCTAGAGATGTGGTGATA	66.8°C
P1-2:	CATACGGAAGAAGACAGAAGACCTGATA	67.8°C
P1-3:	TCATACTCAGGAGTCGAGAACTGAAGATA	67.6°C
P1-4:	CATACTGTGTACGTCAACACGTCAGATA	67.4°C
P1-5:	CATACTGTGGAACTACCATCCAAGGATA	68.0°C
P1-6:	CCATCCAACATCGTTGGAAGAT	67.8°C
P1-7:	CATACAACCTGTCCTGTGAGATCTGATA	67.7°C
P1-8:	ATACTCACGAAGCTGGATGATGAGATA	67.3°C
P1-9:	CATACTAGCATCGATCGAACGTAGGATA	68.1°C
P1-10:	TCATACTCGAAGCTACTGTCGAGATGATA	68.2°C
P2-1:	ATATTAGTGTGTGACGATGGTACGCA	67.8°C
P3-1:	ACAAGTACGAACGTGCATCAGAGA	67.7°C
P4-1:	CGAGCAGGACCTGGAACCT	67.7°C
P4-2:	TCGACCACTGCAGGTGGA	68.3°C

P4-3:	GCTTCCTCTGCTGCACCA	66.7°C
P4-4:	GGTGTCGAGGTGAGCAGCA	69.1°C
P4-5:	CGACGAGGTCCATCCTGGT	68.6°C
P4-6:	GTGAGGAGCAGGTCCTCCTGT	68.0°C
P4-7:	CTGACACTGGTCGTGGTCGA	68.8°C
P4-8:	CATCTCGACGACCTGCTCCT	67.9°C
P4-9:	ACGAGGTCTCCACTGGTCCA	68.3°C
P4-10:	ACTGAGCTGCTCCTCCAGGT	66.5°C

Thermocycling/measurement of fluoresence was performed on an Applied Biosystems ABI Prism 7900HT real-time instrument utilizing the standard cycling parameters:

95°C 10 min;

40 cycles of

95°C 15 sec;

60°C 1 min

All samples were run in duplicate.

Results

Fig. 12 shows the standard curve calculated by the 7900HT system software. The log of the starting copy number was plotted against the measured C_T value. The relationship between C_T and starting copy number was linear in the range from 10 to 10^9 template copies.

This standard curve was utilized by the system software to calculate the quantity in the "unknown" samples as shown below.

Table I: Sample A (Shown graphically in Fig. 13A

Sample A: Equi-molar ratios	Observed A	Observed B	Expected
FPv2	12539947,00	11977503,00	10000000
P1-1	445841,90	480382,03	1000000
P1-2	884840,70	847478,56	1000000
P1-3	1013073,56	948770,00	1000000
P1-4	764187,94	741304,40	1000000
P1-5	1352874,60	1275155,50	1000000
P1-6	1284075,60	1337928,50	1000000
P1-7	658161,80	747371,56	1000000
P1-8	742187,20	653874,00	1000000
P1-9	824587,75	705785,75	1000000
P1-10	813550,75	836037,90	1000000
P2-1	13145159,00	14482606,00	10000000
P3-1	13263911,00	12773780,00	10000000
P4-1	1430704,80	1472576,80	1000000
P4-2	2681652,00	2481824,80	1000000
P4-3	1933106,80	2085476,40	1000000
P4-4	1359684,40	1364621,40	1000000
P4-5	2206709,80	2065813,60	1000000
P4-6	1652718,10	1873777,20	1000000
P4-7	1468208,10	1416153,00	1000000
P4-8	1664467,50	1581067,00	1000000
P4-9	1462520,60	1594593,80	1000000
P4-10	2020088,20	1912277,40	1000000

Table II: Sample B (Shown graphically in Fig. 13B)

Sample B: 2-fold	Observed A	Observed B	Expected

dil.			
FPv2	4,97E+06	5,05E+06	10000000
P1-1	9955,07	10899,97	9765,625
P1-2	12732,32	13469,12	19531,25
P1-3	25542,8	25419,85	39062,5
P1-4	34748,89	44070,81	78125
P1-5	110881,41	123734,13	156250
P1-6	163687,44	166220,5	312500
P1-7	156993,81	172005,64	625000
P1-8	343176,78	374809,13	1250000
P1-9	646619,44	576151	2500000
P1-10	1,49E+06	1,72E+06	5000000
P2-1	5,19E+06	5,37E+06	10000000
P3-1	5,29E+06	5,09E+06	10000000
P4-1	(no signal)	70223,8	9765,625
P4-2	42103,32	22733,17	19531,25
P4-3	54480,62	39663,62	39062,5
P4-4	51293,07	43950,9	78125
P4-5	137946,95	115027,34	156250
P4-6	174134,64	156442,55	312500
P4-7	316505,78	283856,84	625000
P4-8	737661,44	691296,75	1250000
P4-9	1,42E+06	1,45E+06	2500000
P4-10	3,72E+06	3,52E+06	5000000

The results of the experiments show the possibility of accurately quantification of identifier oligonucleotides down to or even below 10 copies with a 9 fold dynamic range, and reliable relative quantification of the tested codons in various positions in the identifier oligonucleotide.

While the invention has been described with references to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention. All patent and literature references cited herein are hereby incorporated by reference in their entirety.

The following items describe embodiments of the first aspect of the present invention:

Item 1: A method for obtaining structural information about an encoded molecule produced by a process comprising reaction of a plurality of chemical entities, said encoded molecule being capable of forming part of a complex also comprising an identifier oligonucleotide containing codons informative of the identity of chemical entities which have participated in the formation of the encoded molecule, the method comprises

a) mixing a primer oligonucleotide with the identifier oligonucleotide,

b) subjecting the mixture to a condition allowing for an extension reaction to occur when the primer is sufficient complementary to a part of the identifier oligonucleotide, and

c) evaluating, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons.

Item 2: The method according to Item 1, wherein a composition of one, two, or more identifier oligonucleotides are processed simultaneously.

Item 3: The method according to Item 2, the composition is a result of subjecting a library of different complexes to a condition partitioning one or more complexes having a certain property from the remainder of the library.

Item 4: The method according to Item 1, wherein the condition which allows for an extension reaction to occur includes a polymerase or a ligase as well as suitable substrates.

Item 5: The method according to Item 4, wherein the condition includes a polymerase and a substrate comprising a blend of (deoxy)ribonucleotide triphosphates.

Item 6: The method according to any of the preceding Items, wherein the chemical entities are precursors for structural units appearing in the encoded molecule.

Item 7: The method according to any of the Items 1 to 6, wherein the process of producing the one or more encoded molecules comprises transferring one or more chemical entities to a nascent encoded molecule by a building block which further comprises an anti-codon.

Item 8: The method of Item 7, wherein the information of the anti-codon is transferred in conjunction with the chemical entity to the nascent encoded molecule.

Item 9: The method according to any of the preceding Items 1 to 8, wherein the identifier comprises two or more codons.

Item 10: The method according to any of the preceding Items 1 to 8, wherein the identifier comprises three or more codons.

Item 11: The method according to any of the preceding Items, wherein neighbouring codons of the identifier are spaced by a framing sequence.

Item 12: The method according to Item 11, wherein the framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule.

Item 13: The method according to any of the Items 1 to 12, wherein at least a part of the primer is complementary to a codon.

Item 14: The method according to any of Items 1 to 13, wherein at least a part of the primer is complementary to a codon and an adjacent framing sequnece.

Item 15: The method according to any of the Items 1 to 13, wherein the codons have a length of four or more nucleotides.

Item 16: The method according to any of the Items 1 to 15, wherein the sequence comprising the codon and an adjacent framing sequence has a total length of 11 nucleotides or more.

Item 17: The method according to any of the Items 1 to 16, wherein the extension reaction is measured using the polymerase chain reaction (PCR), wherein the primer of Item 1 is involved in said PCR.

Item 18: The method according to any of the Items 1 to 17, wherein a primer is labelled.

Item 19: The method according to Item 18, wherein the primer is labelled with a small molecule, a radio active component, or a fluorogenic molecule.

Item 20: The method according to Item 19, wherein the small molecule label is selected from biotin, dinitrophenol, and digoxigenin, and the PCR amplicons are detected using an enzyme labelled streptavidin, anti-dinitrophenol. or anti-digoxigenin, respectively, reporter molecule.

Item 21: The method according to any of the Items 1 to 19, wherein extension reaction is measured by real-time PCR.

Item 22: The method according to Item 21, wherein the real-time PCR involves the use of an oligonucleotide probe responsible for the generation of a detectable signal during the propagation of the PCR reaction.

Item 23: The method according to any of the Items 1 to 21, wherein the probe is designed to hybridise at a position downstream of a primer binding site.

Item 24: The method according to Item 22 or 23, wherein the probe is a 5' nuclease oligoprobe or a hairpin oligoprobe.

Item 25: The method according to Items 2 or 3, wherein the library comprises complexes with identifier oligonucleotides having n codon positions and the codons in said codon positions being selected from a set of m different codons.

Item 26: The method according to Item 25, wherein a framing sequence is related to each of the n codon positions in a particular complex, said framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule.

Item 27: The method according to Item 25, wherein each codon in the set of m different codons differs from any other codons in the set in at least two nucleotide positions.

Item 28: The method according to Item 26, wherein each framing sequence in a set of n different framing sequences differs from any other framing sequences in the set in at least two nucleotide positions.

Item 29: A method for identifying the chemical entities utilized in the formation of an encoded molecule or a composition of encoded molecules, wherein in separate compartments, n x m primers individually are mixed with an aliquot of a composition obtained by subjecting a library of different complexes to a condition partitioning said composition from the remainder of the library, subjected to a mixture of polymerase and substrate (deoxy)ribonucleotide triphosphates under conditions allowing for an extension reaction to occur when a primer is sufficient complementary to a part of one or more identifier oligonucleotides present in the aliquot, and evaluation, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons in each compartment.

Item 30: A set comprising a collection of oligonucleotide primers, a polymerase, a composition of (deoxy)ribonucleotide triphosphates (dNTPs), and a library of complexes composed of a display molecule part and an identifier oligonucleotide, said oligonucleotide comprising codons informative of the identity of the chemical entities which has participated in the formation of the display molecule, wherein the oligonucleotide primers are sufficient complementary to codons appearing on the identifier oligo nucleotides in the library to allow for an extension reaction to occur.

Item 31: An encoded molecule identified by a method according to Items 1 to 28.

Method for identifying a display molecule

The following pages will describe the second aspect of the invention: A METHOD FOR IDENTIFYING A DISPLAY MOLECULE, in which various patent and non-patent references cited in the present application are hereby incorporated by reference in their entirety. It is envisaged that any of the embodiments of the first aspect of the present invention may be used in combination with any of the embodiments of the second aspect of the present invention; indeed any of the features described in relation to the first aspect of

the present invention may be used in combination with any of the embodiments or features of the second aspect of the present invention, and vice versa.

The second aspect of the present invention relates to a method for identifying from a library a display molecule having affinity towards one or more molecular targets. The display molecule is a part of a complex also comprising an identifier oligonucleotide that codes for said display molecule.

Traditional drug discovery begins with a pathological phenomenon in an organism and the development of a therapeutic theory to combat this. A chemical concept follows to produce compounds for screening. Most of the processes for curing the pathological phenomenon originate with the understanding of some biological pathways and screening for an effect in tissues or cells. This may or may not eventually reveal a "target". The target can be identified by various conventional methods, including protein expressing, protein chemistry, structure-functional studies, knowledge of biochemical pathways, and genetic studies.

In recent years, genetic information has increasingly guided the identification of molecular targets. These are derived from the knowledge of the genes of specific cell phenotypes that encode proteins that may be involved in the pathogenesis of a particular disease state.

A lead is a compound, usually a small organic molecule that demonstrates a desired biological activity on a target. Usually, a collection of compounds, referred to as a "library", is screened before a useful lead is identified. Today, many libraries are commercially available or open to public. Most pharmaceutical companies house their own compilation of compounds that have been synthesised over several years and screened against a variety of targets.

Each compound in a library must be screened by an appropriate assay against the target. Techniques for handling the screening of several thousands compounds simultaneously have been developed and are generally referred to as high-throughput screening techniques. To push the limit of compounds possible to screen simultaneously, different manufactures have been developing instrumentation capable of handling multiple micro titer plate formats on the same platform using 384 and 1536-well plates. Advances in small volume liquid dispensing and pipetting, reliable handling of standardized plates and simplified assay formats all have made an impact on the reliability of the high-throughput screening process.

However, high-throughput screening has the disadvantage that each of the compounds has to be positioned in spatially discrete regions, usually in wells of a micro titer plate in order to observe an interaction with a target. If more than a single compound is present, it is not feasible to discern which compound displaying the appropriate biological activity. Thus, the full power of combinatorial chemistry cannot be applied because a collection of compounds usually is produced in a single container.

To be able to select a possible lead compound in a collection of compounds placed in the same container, libraries of bifunctional complexes have been evolved. Each bifunctional complex in the library comprises a potential lead compound coupled to an identifier oligonucleotide sequence. The identifier oligonucleotide sequence is suitably a nucleic acid which identifies the potential lead compound. When a library of bifunctional complexes is screened against a target, one or more of the potential leads may bind to the target. After removal of the remainder of the library, the binding bifunctional complexes can be eluated and the lead compound identified by sequencing the identifier oligonucleotide.

Various techniques for producing bifunctional complexes are known from the prior art. Some attempts to form the complex comprising a molecule as well

as the identifier oligonucleotide that codes therefore, are based on the split-and-mix principle known from combinatorial chemistry, see e.g. WO 93/06121 A1, EP 643 778 B1, and WO 00/23458.

Other attempts have focussed on the formation of encoded proteins using the natural machinery of a cell and connecting the formed protein with the template nucleic acid that has coded for the amino acid components of the protein. Examples of suitable systems are phage display, *E. coli* display, ribosome display (WO 93/03172), and protein-mRNA-fusions (WO 98/31700). The genetic information of the nucleic acid, usually mRNA or DNA, may not necessarily be decoded between each round of selection to establish the identity of the chemical entities that has formed the protein because the nucleic acid can be amplified by known means, such as PCR, and processed for the formation of a new library enriched in respect of suitable binding proteins.

Recently, a method for encoding molecules has been suggested, which can be performed in several selection rounds without intermediate decoding, wherein the encoded molecule is not restricted to peptides and proteins. WO 02/00419 and WO 02/103008 disclose methods for preparing virtually any molecule connected to an identifier oligonucleotide coding for chemical entities which have reacted to form the display molecule. In short, a template segregated into a plurality of codons and a plurality of building blocks comprising a transferable chemical entity and an anticodon are initially provided. Under hybridisation conditions, the template and building blocks are annealed together and the chemical entities are subsequently reacted to form the molecule.

The present invention aims at providing an efficient method for identifying display molecules having affinity towards a target using a library of bifunctional complexes.

The second aspect of the present invention concerns a method for identifying display molecule(s) having affinity towards molecular target(s), comprising the steps of

mixing one or more molecular target(s) associated with target oligonucleotide(s) and a library of bifunctional complexes, each bifunctional complex of the library comprising a display molecule attached to an identifier oligonucleotide, which codes for said display molecule,

coupling to the target oligonucleotide(s) the identifier oligonucleotide of complexes comprising display molecules binding to the target, and

deducing the identity of the binding display molecule(s) and/or the molecular target(s) from the coupled product between the identifier oligonucleotide(s) and the target oligonucleotide(s).

The second aspect of the present invention is based on the realization that a close proximity of the identifier oligonucleotides relative to the target oligonucleotide is obtained when a display molecule has binding affinity towards a target and therefore, will be more prone to be coupled to the target oligonucleotide compared to identifier oligonucleotides of complexes not comprising a display molecule having affinity toward the target. The tendency to be coupled together depends on various factors such as (1) the affinity of the displayed molecule towards the target, (2) the length between the displayed molecule and the coupling point on the identifier oligonucleotide sequence as well as the length between the target molecule and the coupling point on the target sequence, and (3) possible steric effects resulting from the nature of the target or the oligonucleotides. The proximity effect, and thus the power of selectivity, will increase with higher local concentration of ends of nucleotides to be coupled together. Thus, various embodiments of the present invention may be envisaged to fine-tune the local concentration of the ends of the oligonucleotides, including appropriately selected lengths of target and identifier oligonucleotides, site of attachment of the target oligonucleotide to the target relative to the binding site of the display molecule, and size of target.

The molecular target may be of a biological origin or may be a synthetic molecular target. Typically, the molecular target stems from an organism selected from human and animals, especially vertebras. However, in other embodiments the target may originate from a plant. In the quest for a compound with therapeutical effect on the human or animal body, the target is usually expected to have an importance in a therapeutically theory that combats a certain disease. In the quest for discovering compounds with plant protective effect, the target is usually expected to originate from an organism that harms the crop or a competing undesired plant. The organism may be a fungus when a compound with fungicide effect is searched for or an insect when a compound having insecticide effect is desired. Optionally, a protein target stemming from a biological origin may be derivatised by altering, adding, or deleting one or more amino acids.

The molecular target may be a protein, a small molecular hormone, a lipid, a polysaccharide, a whole cell, a nucleic acid, a metabolite, a heme group, etc. In a preferred aspect the target is a protein. The protein may serve the function in the organism of being an enzyme, a hormone, a structural element, a regulatory protein, a membrane channel or pump, a part of a signal transducing cascade, an antibody, etc. Suitable target enzymes include kinases, phosphatates, and proteases. The protein may occur as an independent entity or may be dimers, trimers, tetramers, or polymers and the protein may comprise a prosthetic group. Also, the target may be a fusion protein having two or more functionalities. Furthermore, the molecular target may be a soluble or insoluble agglomerate of one or more proteins and one or more substituents occurring in the body or artificial components. In another preferred embodiment, the molecular target is a nucleic acid, such as DNA or RNA aptamer or ribozyme.

The target may be immobilized to a support or be present as a solution or a emulsion, as appropriate. The target optionally immobilized on the support, may also form a stable or quasi-stable dispersion in the media. In a certain

embodiment, the target is in solution and all the reactions occur in the solution too. The absence of an immobilization step reduces the background noise because there is no background surface to associate to. Thus the result of the assay may be more sensitive. In solution, the only background noise imaginable is when the oligonucleotides or display molecules of the library of complexes binds unspecific to the target molecules or the target oligonucleotides. The absence of an immobilization step generally necessitates a subsequent recovery step, eg. by chromatography.

In certain aspects of the invention, it is preferred to immobilize the molecular target on a solid support. The solid support may be beads of a column or the surface of a container. The immobilisation of the molecular target may ease the removal of the non-binding complexes by washing or similar means. In a certain embodiment, a cleavable linkage between the molecular target and the solid support is present. The cleavable linker is preferably selectively cleavable, that is, the linkage can be cleaved without cleaving other linkages in the target or the complexes. The cleavage of the linkage between the molecular target and the solid support may reduce the contribution from the background, such as complexes associated with the surface of the solid support and not binding to the molecular target.

A single or a multitude of different molecular targets may be mixed with the library of complexes. If two or more different targets are mixed with the library of complexes it is appropriate to provide the molecular targets with a genetic sequence coding for the identity of the target in question. Proving the targets with identifying oligonucleotides allows for a simultaneous decoding of the binding partners, i.e. the molecular target and the display molecule. The simultaneous decoding is not only suitable for finding binding partners. It is also valuable for finding a possible cross-binding interaction or to find other display molecules competing for the same target. Furthermore, appropriate selection of display molecules and/or molecular targets, can generate useful

information for preparing a structure-activity relationship (usually abbreviated SAR).

The molecular target may be obtained in any suitable way. A variety of targets are commercially available, either as purified protein or as the corresponding cDNA. Other protein or peptide targets may be isolated from tissues or mRNA (or the corresponding cDNA) may be extracted from a tissue. Smaller peptides may be synthesised chemically using the standard solid-phase Fmoc peptide synthesis. When nucleic acids are used or included in the molecular target, it may be synthesised using the standard amedite synthesis method or by using the natural machinery.

The target can be associated with the target oligonucleotide using any suitable means and the association may involve a covalent or non-covalent linkage. In an aspect of the invention the oligonucleotide is associated with the target utilizing a chemical synthesis. A protein usually comprises several groups that may be functionalized and used as attaching point. As examples the side chain of lysine contains an amino group, the side chain of serine contains a hydroxyl group and the side chain of cystein contains a thiol group, all of which may serve as anchoring point for a target oligonucleotide comprising e.g. a carboxylic acid group. In another aspect of the invention, the protein target is fused to a tag, such as a His-tag, Flag-tag, antibody, or streptavidin. The tag can be selectively recognized by an anti-body or small molecule such as biotin or dinitrophenol. The anti-body or the small molecule is attached to the target oligonucleotide, thereby ensuring an efficient coupling of the molecular target to the target oligonucleotide.

The target oligonucleotide can be associated to the molecular target through a cleavable linker. The cleavable linkage can be used to separate the target oligonucleotide from the molecular target or the coupled product from the target at a point in time following the contacting between the target and library of complexes. The target oligonucleotide can be distanced from the

molecular target by a suitable linker, such as a polyethylene linker or a non-coding nucleotide sequence. A linker may remedy any interaction that possible can be between the molecular target and the target oligonucleotide and at the same time provide suffice space for an enzyme to perform its action.

A variety or methods for association of an oligonucleotide to a target is available for the skilled person in the art. An option involves the association of a target protein with the mRNA responsible for the formation thereof. This method is generally referred to as mRNA display. Optionally, the mRNA may be substituted with the corresponding cDNA. A method for generation such a single or a library of fusions between a protein and the mRNA responsible for the formation thereof is disclosed in WO 98/31700. The corresponding DNA strand may be attached to the protein using the method disclosed in WO 00/32823. The contents of both patent applications being incorporated in their entirety by reference herein. The method of WO 98/31700 includes providing a RNA stand comprising a translation initiation sequence, a start codon operable linked to a protein encoding sequence, and a peptide acceptor at the 3' end and translating the protein encoding sequence to produce a RNA-protein fusion. According to WO 00/32823 a DNA primer is covalently connected to the 3' end of the mRNA strand and extended by reverse transcriptase a to prepare the complementing DNA strand. The original RNA strand may be digested by RNase H. Another suitable method for generating a target library is disclosed in WO 01/90414, the content of which is incorporated herein by reference.

In accordance with another option, the target oligonucleotide is associated with a target using a method generally referred to as ribosome display. Ribosome display is disclosed in WO 93/03172, the content of which is included herein by reference. A still further option for association is a variation of the phage display, in which a target is displayed on the capsule of the phage and a target oligonucleotide is connected to the same capsule. Suitably, the target oligonucleotide is connected to the capsule via reactive groups positioned

on proteins expressed on the capsule. A suitable reactive group is -SH emanating from cystein.

A further option for associating the target with an oligonucleotide includes the method disclosed in M. Yonezawa *et al*, Nucleic acid research, 2003, vol. 31, No. 19 e118 (included by reference). The method includes the initial provision of a target oligonucleotide connected to biotin and compartmentalization thereof together with a transcription and translation system. The target oligonucleotide comprises a fusion gene coding for streptavidin and a target. After the formation of the fusion protein in each compartment, the streptavidin part of the fusion protein binds to the biotin moiety of the target oligonucleotide, thereby associating the target with the target oligonucleotide.

In case the target is a nucleic acid, it may be of the aptamer type, i.e. an aptamer or a library of aptamers comprising constant nucleic acid regions flanking a random oligonucleotide part. The random oligonucleotide part serves the function of a molecular target in the present invention and one or both the constant region serves as target oligonucleotides. Alternatively, an additional oligonucleotide sequence can be adhered to one or both the constant regions to serve as the target oligonucleotide. The present invention also allows for the selection of pairs of aptamers that either binds to each other or binds to the same target but to different epitopes. The latter embodiment is of particular relevance when evolving detection assays, where aptamers that bind to different epitopes on the same target may be desired.

In certain embodiments, a bifunctional complex having a display molecule binding to the molecular target constitutes the target oligonucleotide associated with the molecular target. The bifunctional complexes binding to the target and serving to associate an oligonucleotide to the molecular target may be provided prior to or during with the mixing step. In case the target oligonucleotide is associated with the molecular target during the mixing step the bifunctional complex may be a member of the library or may be a complex

added to the mixture. In some aspects of the invention, the display molecule is a compound known to bind to the target. The display molecule may be known to bind to the target from the prior art or from preceding screening procedures. To find a second or further binding compound, the target is generally saturated with the known display molecule prior to the mixing step. The known binding molecule may or may not be attached to a nucleic acid sequence.

In case the target oligonucleotide emanates from an identifier oligonucleotide in the library of complexes, two bifunctional complexes of a library of bifunctional complexes are associated with a common molecular target and can be discovered simultaneously. The bifunctional complexes may bind to the same binding site of the molecular target or the bifunctional complexes may bind to different binding sites. In case the bifunctional complexes bind to the same binding sites, the display molecules may be connected to each other through a covalent or non-covalent chemical bond, e.g. by a technique known as click chemistry. By way of example only, the two display molecules can be connected through a disulphide bridge. In aspects of the invention in which the display molecules binds to discrete binding domains on the same target molecule they are usually adhered together after the identification process of the present invention by a suitable linker in order to form the effective compound.

In a certain aspects of the invention an iterative method for finding the desired compound is applied. An initial display molecule or a pool of display molecules with affinity towards a target is found using the present method or another method. In the event the affinity is in the lower range of what is desired, the initial display molecule is amended by reaction with one or more chemical entities to form a second generation library, said second generation library being used in the method of the present invention.

In Nature, biochemical components interact. In a certain aspect of the present invention, two or more targets interacting in a biological context are separately subjected to the method of the invention, whereupon the identified display molecules binding to the two or more targets are linked via a suitable linker. By way of example only, blood factors, such as factor VIIa and Xa may be prevented or promoted in the their interaction with each other using a compound having a part binding to a first blood factor and a part binding to a second blood factor. In other aspects of the invention, targets are linked by a suitable linker, as disclosed in Fig. 10.

A library of molecular targets may be generated by starting out from a library of DNA molecules, usually cDNA molecules, and preparing the corresponding RNA strands by a suitable RNA polymerase. In according with a certain aspect of the invention, the mixture step therefore includes that a molecular target library comprising different peptides each attached to the nucleic responsible for the formation thereof is mixed with a library of complexes.

The coupling of the target oligonucleotide and the identifier oligonucleotide is promoted due to the relative high local concentration of the ends of oligonucleotides. The complexes in solution, i.e. complexes not bund to the target, are relatively remote from the target oligonucleotide and the tendency of the target oligonucleotides to be coupled to such unspecific complexes is reduced. Among other things, the unspecific coupling depends on the concentration of the complexes in solution.

In an aspect of the invention the non-binding library members are either diluted or at least partly removed prior to the coupling step. Dilution may be performed by adding a suitable buffer and the removal may be preformed by washing one or more times with a suitable liquid, such as a buffer. In the event the target molecule is immobilized on a bead, the beads may be retained on a filter while non-binding complexes is transported through the filter. In case the target is in solution or made in solution by cleavage of a bond

immobilising the target, optional following one or more washing step, the removal can be performed by chromatography, such as size-exclusion chromatography.

Thus, in a certain aspect of the invention, the mixing step includes the removal of non-binding library members prior to the coupling of the target oligonucleotide and the identifier nucleotide together.

It may be an advantage to have all or at least a part of the nucleotides on a double stranded form during the contacting with the molecular target, as certain nucleic acids may perform a binding interaction or a catalytical action on the components present during the mixing step. Thus, in one embodiment of the invention, the target oligonucleotide and/or the identifier oligonucleotide partly or fully is hybridised to a complementing oligonucleotide.

The coupling may be performed using any suitable means that ensures a physical connection. Suitably, the coupling is performed using means selected from the group consisting of chemical means, enzymatic means, and design means.

The chemical means for coupling the ends together can be selected from a large plethora. Suitable examples include that
a) a first oligonucleotide end comprises a 3'-OH group and the second oligonucleotide end comprises a 5'-phosphor-2-imidazole group. When reacted a phosphodiester internucleoside linkage is formed,
b) a first oligonucleotide end comprising a phosphoimidazolide group and the 3'-end and a phosphoimidazolide group at the 5'-and. When reacted together a phosphodiester internucleoside linkage is formed,
c) a first oligonucleotide end comprising a 3'-phosphorothioate group and a second oligonucleotide comprising a 5'-iodine. When the two groups are reacted a 3'-O-P(=O)(OH)-S-5' internucleoside linkage is formed, and

d) a first oligonucleotide end comprising a 3'-phosphorothioate group and a second oligonucleotide comprising a 5'-tosylate. When reacted a 3'-O-P(=O)(OH)-S-5' internucleoside linkage is formed.

Suitably, the target oligonucleotide or a complementing target oligonucleotide and the indentifier oligonucleotide or a complementing identifier oligonucleotide operatively are joined together, so that as to allow a nucleic acid active enzyme to recognize the coupling area a substrate. Notably, in a preferred embodiment, the coupling is performed so as to allow a polymerase to recognise the coupled strand as a template. Thus, in a preferred aspect, a chemical reaction strategy for the coupling step generally includes the formation of a phosphodiester internucleoside linkage. In accordance with this aspect, method a) and b) above is preferred.

The enzymatic means is in some instances preferred because the coupling reaction is specific, i.e. the risk of side reactions in virtually not present. The enzymatic means is in general selected from the enzymes of the polymerase type, ligase type, and restriction enzymes, as well as any combination thereof.

Ligases are useful means for the coupling step. Suitable examples include Taq DNA ligase, T4 DNA ligase, T7 DNA ligase, and *E. coli* DNA ligase. The choice of the ligase depends to a certain degree on the design of the ends to be joined together. Thus, if the ends are blunt, T4 DNA ligase may be preferred, while a Taq DNA ligase may be preferred for a sticky end ligation, i.e. a ligation in which an overhang on each end is a complement to each other.

In a certain aspect of the invention, a connector oligonucleotide is used. The connector oligonucleotide has a region complementing a distal part of the target oligonucleotide and a region complementing a distal part of the identifier oligonucleotide. If the ends of the target and identifier oligonucleotides abut each other a ligase can ligate the ends together. If a gap exists between

the ends, a polymerase may be used to fill the gap and a ligase may subsequently perform a ligation. The regions of the connector oligonucleotide complementing the identifier oligonucleotide and target oligonucleotide, respectively, may independently be chose, e.g. in the range of 6 to 16 nucleotides, preferably in the range of 8 to 12 nucleotides. In a particular aspect of the invention the connector oligonucleotide is added in excess relative to the total target and identifier oligonucleotides to saturate the ends of complexes not bound to a target.

In another aspect of the invention the coupling is performed by design means. As an example, the regions at the distal ends of the target and identifier oligonucleotides are designed to be complementary to each other. Under hybridisation conditions polymerase is then allowed to extend the target oligonucleotide as well as the identifier oligonucleotide to obtain a double stranded coupled product.

Still another coupling means include the design of the ends of one or more oligonucleotides with sticky ends. In a certain aspect, the target oligonucleotide and/or the identifier oligonucleotide is provided with a sticky end to allow a ligase or a polymerase or a mixture thereof to adjoin the oligonucleotides. Suitably, the sticky ends can be formed by a restriction nuclease. In a practical approach, the target and the identifier oligonucleotides are initially double stranded and provide at the ends with a restriction site. Following the initial contact, the mixture is treated with a restriction nuclease to form the sticky ends. After subsequent removal of the restriction enzyme from the mixture, a ligase is allowed to perform its enzymatic action to form a ligation product.

The coupled product of the identifier and target oligonucleotides comprises the information necessary for decoding the identity of the display molecule and optionally also of the molecular target. The coupled product may be analysed directly in some instances to reveal the identity of the display molecules that have performed an interaction with the molecular target. As an example,

the coupled product may be detached from the target-display molecule interaction using a cut by a restriction nuclease at positions of the coupled product flanking the informative part. The informative part can be decoded in a standard sequencing machine. In general however, it is preferred to include the informative part of the coupled product in to a suitable vector and transfer the vector to a host organism. The host organisms may then be cultivated on a suitable substrate and allowed to form colonies. Samples from the colonies may be used for sequencing in a sequencing machine.

In another approach, the target and/or the identifier oligonucleotides or sequences complementary thereto at the proximal end is provided with a priming site. The priming site may be used for annealing a primer to allow a polymerase to extent the primer using the coupled product as template. Appropriately, the extended strand is designed with another priming site which may allow a second (reverse) primer to anneal thereto and subsequently a polymerase to perform an extension of the primer to produce a sequence identical to the coupled product. The extension product or the amplification product may be analysed directly or be incorporated into a vector which subsequently is transformed into a host organism as explained above. In a certain aspect of the invention, the coupled oligonucleotide is amplified by PCR using priming sites positioned proximal to the display molecule and the molecular target, respectively, and flanking the informative part of the coupled product.

Following the coupling step, the target-display molecule conjugate may be recovered. Any method that result in a recovery may in principle be used, including filtering, washing, elution, chromatography, etc. In a preferred aspect the target-display molecule conjugate is recovered by chromatography following the coupling of the target and the identifier oligonucleotides. Optionally, the various recovering methods may be combined. As an example, a washing or elution step may precede the chromatography step. A presently preferred chromatography method is size-exclusion chromatography. The chromatography is usually performed on a sample in which the target-display

molecule conjugate is in solution. In one aspect of the invention, the target has been cleaved from a solid support prior to the chromatography step.

In an aspect of the invention, selective cleavable chemical moieties at each end of the coupled oligonucleotides are cleaved to liberate the coupled oligonucleotides prior to amplification. Usually, the cleavage is preceded of a step removing the non-binding complexes. The liberated product may be recovered as described above and subjected to amplification.

The invention also pertains to a conjugate comprising a molecular target associated with an oligonucleotide and a bifunctional complex comprising a display molecule attached to an identifier oligonucleotide, which codes for said molecule. Usually, the display molecule part of the complex is bound to the target. The target oligonucleotide and/or the identifier oligonucleotide of the conjugate are in a certain embodiment joined to the molecular target and/or the display molecule, respectively, through a selectively cleavable linkage. In an embodiment of the invention, the target oligonucleotide and the identifier oligonucleotide are coupled together, whereby a product is obtained wherein a display molecule is bound to the molecular target and the identifier and target oligonucleotides are coupled together. In a certain aspect, the coupled oligonucleotide is amplifiable. The amplifiability is usually obtained through a coupling that involves a phospodiester internucleoside linkage.

The present invention also extends to a display molecule identified by any of the methods disclosed herein.

Detailed Description

Complex

The complex used in the present invention comprises a display molecule and an identifier oligonucleotide. The identifier oligonucleotide comprises identifying moieties that identifies the display molecule. Preferably, the identifier oligonucleotide identifies the molecule uniquely, i.e. in a library of complexes a

particular identifier oligonucleotide is capable of distinguishing the molecule it is attached to from the rest of the display molecules.

The display molecule and the identifier oligonucleotide may be attached directly to each other or through a bridging moiety. In one aspect of the invention, the bridging moiety is a selectively cleavable linkage.

The method may in certain embodiments be performed without amplification after the coupling step. However, when larger libraries are used and the amount of separated coupled product oligonucleotide is relatively low, it is in general preferred to use an identifier oligonucleotide and a coupled prodct which is amplifiable. Identifier oligonucleotides comprising a sequence of nucleotides may be amplified using standard techniques, like PCR.

The identifier oligonucleotide may comprise two or more codons. The sequence of codons can be decoded to identify reactants used in the formation of the molecule. When the identifier oligonucleotide comprises more than one codon, each member of a pool of chemical entities can be identified and the order of codons is informative of the synthesis step each member has been incorporated in.

The sequence of the nucleotides in each codon may have any suitable length. The codon may be a single nucleotide or a plurality of nucleotides. In some aspects of the invention, it is preferred that each codon independently comprises four or more nucleotides, more preferred 4 to 30 nucleotides.

The identifier oligonucleotide will in general have at least two codons arranged in sequence, i.e. next to each other. Two neighbouring codons may be separated by a framing sequence. Depending on the encoded molecule formed, the identifier oligonucleotide may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a suitable framing sequence. Preferably, all or at least a majority of the codons

of the identifier oligonucleotide are separated from a neighbouring codon by a framing sequence. The framing sequence may have any suitable number of nucleotides, e.g. 1 to 20. Alternatively, codons on the identifier oligonucleotide may be designed with overlapping sequences.

The framing sequence, if present, may serve various purposes. In one setup of the invention, the framing sequence identifies the position of the codon. Usually, the framing sequence either upstream or downstream of a codon comprises information which allows determination of the position of the codons. In another setup, the frames have alternating sequences, allowing for addition of building blocks from two pools in the formation of the library. The framing sequence may also or in addition provide for a region of high affinity. The high affinity region may ensure that the hybridisation of the template with an anti-codon will occur in frame. Moreover, the framing sequence may adjust the annealing temperature to a desired level.

A framing sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. Examples of nucleobases having this property are guanine and cytosine. Alternatively, or in addition, the framing sequence may be subjected to backbone modification. Several back bone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

The identifier oligonucleotide may comprise one or two flanking regions. The flanking region can encompass a signal group, such as a flourophor or a radio active group to allow for detection of the presence or absence of a complex or the flanking region may comprise a label that may be detected, such as biotin. When the identifier oligonucleotide comprises a biotin moiety, the identifier oligonucleotide may easily be recovered.

The flanking region(s) can also serve as priming sites for amplification reactions, such as PCR. The identifier oligonucleotide may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block.

It is to be understood that when the term identifier oligonucleotide is used in the present description and claims, the identifier oligonucleotide may be in the sense or the anti-sense format, i.e. the identifier oligonucleotide can be a sequence of codons which actually codes for the molecule or can be a sequence complementary thereto. Moreover, the identifier oligonucleotide may be single-stranded or double-stranded, as appropriate.

The display molecule part of the complex is generally of a structure expected of having an effect on the target. When the target is of pharmaceutical importance, the molecule is generally a possible drug candidate. The complex may be formed by tagging a library of different possible drug candidates with a tag, e.g. a nucleic acid tag identifying each possible drug candidate. In another embodiment of the invention, the molecule is encoded, i.e. formed by a variety of reactants which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the final molecule displayed on the complex. The post-modification may involve the cleavage of one or more chemical bonds attaching the encoded molecule to the indentifier in order more efficiently to display the encoded molecule. In still another embodiment the display molecule is a polypeptide formed using the natural machinery, such as the methods disclosed in WO 92/02536, WO 91/05058, and US 6,194,550.

The formation of a synthetic encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may react with a reactive group also appearing on the original scaffold or a

reactive group incorporated by the first chemical entity. Further chemical entities may be involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule may be mediated by a bridging molecule. As an example, if the nascent encoded molecule and the chemical entity both comprise an amine group a connection between these can be mediated by a dicarboxylic acid. A display molecule is in general produced in vitro and may be a naturally occurring or an artificial substance. In an aspect of the invention, a display molecule is not produced using the naturally translation system in an in vitro process. In other aspects of the invention, the display molecule is a polypeptide produced using the natural translation machinery.

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading to the final display molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiments the building blocks also comprise an affinity region providing for affinity towards the nascent complex.

Thus, the chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anti-codon serves the function of transferring the genetic information of the building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order, however, it is important that a correspondence is maintained in the complex. The chemical entities are preferably reacted without enzymatic interaction in some aspects of the invention. Notably, the reaction of the chemical entities is preferably not mediated by ribosomes or enzymes having similar activity. In another aspect of the invention a ribosome is used to translate an mRNA into a protein using a tRNA loaded with a natural or unnatural amino acid. In still another aspect of the invention, enzymes having catalytic activities

different from that of ribosomes are used in the formation of the display molecule.

According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on a nucleic acid template. Another method for transferring the genetic information of the anti-codon to the nascent complex is to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the genetic information of the anti-codon to the nascent complex by an extension reaction using a polymerase and a mixture of dNTPs.

The chemical entity of the building block may in certain cases be regarded as a precursor for the structural entity eventually incorporated into the encoded molecule. In other cases the chemical entity provides for the eliminations of chemical units of the nascent encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is reacted with a nascent encoded molecule it is to be understood that not necessarily all the atoms of the original chemical entity is to be found in the eventually formed encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The number of reactive groups which appears on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used *i.a.* in the end positions of

polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. Non-limiting examples of scaffolds are opiates, steroids, benzodiazepines, hydantoines, and peptidylphosphonates.

The reactive group of the chemical entity may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a chemical reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In some aspects of the invention, it is appropriate to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and

cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule.

The display molecules of the invention may have any chemical structure. In a preferred aspect, the display molecule can be any compound that may be synthesized in a component-by-component fashion. In some aspects the display molecule is a linear or branched polymer. In another aspect the display molecule is a scaffolded molecule. The term "display molecule" also comprises naturally occurring molecules like α-polypeptides etc, however produced *in vitro* usually in the absence of enzymes, like ribosomes. In certain aspects, the display molecule of the library is a non-α-polypeptide.

The display molecule may have any molecular weight. However, in order to be orally available, it is in this case preferred that the display molecule has a molecular weight less than 2000 Daltons, preferably less than 1000 Dalton, and more preferred less than 500 Daltons.

The size of the library may vary considerably pending on the expected result of the inventive method. In some aspects, it may be sufficient that the library

comprises two, three, or four different complexes. However, in most events, more than two different complexes are desired to obtain a higher diversity. In some aspects, the library comprises 1,000 or more different complexes, more preferred 1,000,000 or more different complexes. The upper limit for the size of the library is only restricted by the size of the vessel in which the library is comprised. It may be calculated that a vial may comprise up to 10^{14} different complexes.

Methods for forming libraries of complexes

The complexes comprising an identifier oligonucleotide having two or more codons that codes for reactants that have reacted in the formation of the molecule part of the complex may be formed by a variety of processes. Generally, the preferred methods can be used for the formation of virtually any kind of encode molecule. Suitable examples of processes include prior art methods disclosed in WO 93/20242, WO 93/06121, WO 00/23458, WO 02/074929, and WO 02/103008, the content of which being incorporated herein by reference as well as methods of the present applicant not yet public available, including the methods disclosed in DK PA 2002 01955 filed 19 December 2002, and DK PA 2003 00430 filed 20 March 2003. Any of these methods may be used, and the entire content of the patent applications are included herein by reference.

Below four preferred embodiments are described. A first embodiment disclosed in more detail in WO 02/103008 is based on the use of a polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides is provided. Subsequently primers are annealed to each of the templates and a polymerase is extending the primer using nucleotide derivatives which have appended chemical entities. Subsequent to or simultaneously with the incorporation of the nucleotide derivatives, the chemical entities are reacted to form a reaction product. The encoded molecule may be post-modified by cleaving some of the linking moieties to better present the encoded molecule.

Several possible reaction approaches for the chemical entities are apparent. First, the nucleotide derivatives can be incorporated and the chemical entities subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide bond. Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such as an ester or a thioester group. An adjacent building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

A second embodiment for obtainment of complexes pertains to the use of hybridisation of building blocks to a template and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed such that they recognise a sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codon and the codon to each other a reaction of the chemical entity is effected.

The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

A third embodiment for the generation of a complex includes chemical or enzymatical ligation of building blocks when these are lined up on a template.

Initially, templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product. The method is disclosed in more detail in DK PA 2003 00430 filed 20 March 2003.

A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region is annealed to a building block comprising a region complementary to the affinity section. Subsequently the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon. This method is disclosed in detail in DK PA 2002 01955 filed 19 December 2002 and DK PA 2003 01064, filed 11 July 2003.

Thus, the codons are either pre-made into one or more templates before the encoded molecules are generated or the codons are transferred simultaneously with the formation of the encoded molecules.

After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however at least one linker must be maintained to provide for the complex.

Nucleotides

The nucleotides used in the present invention may be linked together in a sequence of nucleotides, i.e. an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a back-bone. The back bone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosin, N^6,N^6-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C^3-C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

Examples of suitable specific pairs of nucleobases are shown on p27 of this application, entitled "Natural base pairs", "synthetic base pairs", "synthetic purine bases pairing with natural pyrimidines".

Suitable examples of backbone units are shown diagrammatically on p28 of this application (B denotes a nucleobase). The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-flour-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2-deoxyribose. The internucleoside linkage may be

the natural occurring phospodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as universal base because inosine can pair nearly isoenergetically with A, T, and C. Other compounds having the same ability of non-specifically base-pairing with natural nucleobases have been formed. Suitable compounds which may be utilized in the present invention includes among others the compounds depicted on p30 of this application, entitled "Examples of Universal Bases".

Building block

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A

building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed

such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

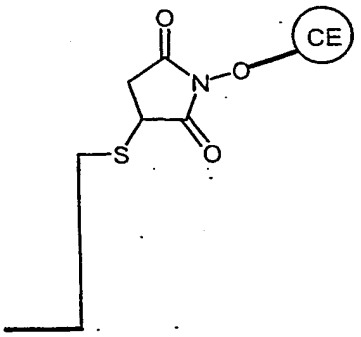
The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule or reactive site.

The anticodon complements the codon of the identifier oligonucleotide sequence and generally comprises the same number of nucleotides as the codon. The anticodon may be adjoined with a fixed sequence, such as a sequence complementing a framing sequence.

Various specific building blocks are envisaged. Building blocks of particular interest are shown below.

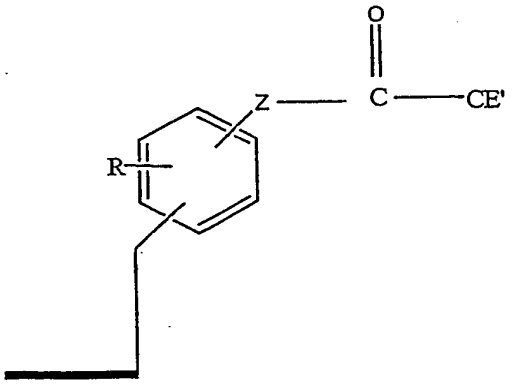
Building blocks transferring a chemical entity to a recipient nucleophilic group

The building block indicated below is capable of transferring a chemical entity (CE) to a recipient nucleophilic group, typically an amine group. The bold lower horizontal line illustrates the building block and the vertical line illustrates a spacer. The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold



The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold, to transfer the chemical entity to the scaffold, thus converting the remainder of the fragment into a leaving group of the reaction. When the chemical entity is connected to the activator through an carbonyl group and the recipient group is an amine, the bond formed on the scaffold will an amide bond. The above building block is the subject of WO03078627A2, the content of which is incorporated herein in their entirety by reference.

Another building block which may form an amide bond is

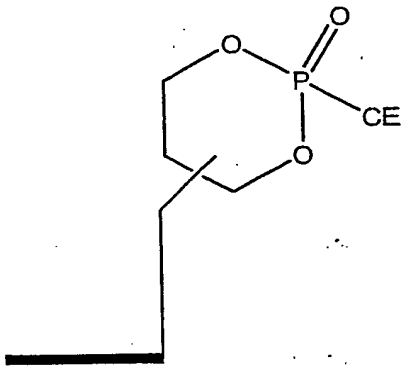


R may be absent or NO_2, CF_3, halogen, preferably Cl, Br, or I, and Z may be S or O. This type of building block is disclosed in WO03078626A2. The content of this patent application is incorporated herein in the entirety by reference.

A nucleophilic group can cleave the linkage between Z and the carbonyl group thereby transferring the chemical entity –(C=O)-CE' to said nucleophilic group.

Building blocks transferring a chemical entity to a recipient reactive group forming a C=C bond

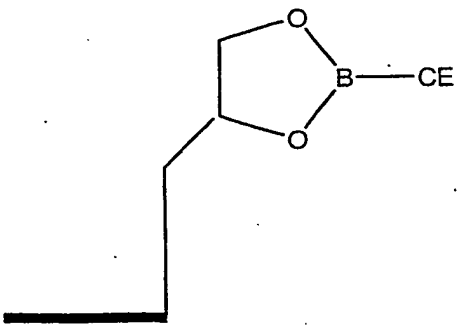
A building block as shown below are able to transfer the chemical entity to a recipient aldehylde group thereby forming a double bond between the carbon of the aldehyde and the chemical entity



The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

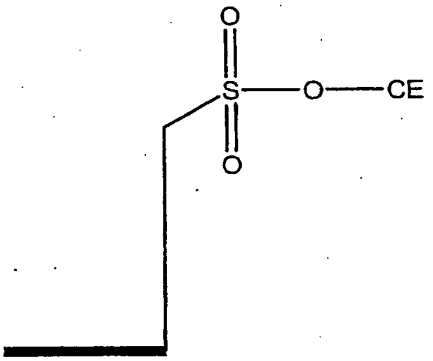
Building blocks transferring a chemical entity to a recipient reactive group forming a C-C bond

The below building block is able to transfer the chemical entity to a recipient group thereby forming a single bond between the receiving moiety, e.g. a scaffold, and the chemical entity.



The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

Another building block capable of transferring a chemical entity to a receiving reactive group forming a single bond is



The receiving group may be a nucleophile, such as a group comprising a hetero atom, thereby forming a single bond between the chemical entity and the hetero atom, or the receiving group may be an electronegative carbon atom, thereby forming a C-C bond between the chemical entity and the scaffold. The above building block is disclose in WO03078446A2, the content of which is incorporated herein by reference.

The chemical entity attached to any of the above building blocks may be a selected from a large arsenal of chemical structures. Examples of chemical entities are

H or entities selected among the group consisting of a C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroal-

kyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-$NR^4{}_2$, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-$NR^4{}_2$, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9.

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from -N_3, -CNO, -$C(NOH)NH_2$, -NHOH, -$NHNHR^6$, -$C(O)R^6$, -$SnR^6{}_3$, -$B(OR^6)_2$, -$P(O)(OR^6)_2$ or the group consisting of C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and

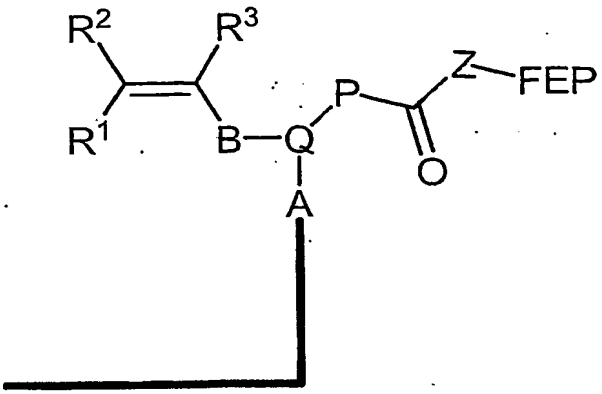
R^7 is independently selected from –NO_2, -$COOR^6$, -COR^6, -CN, -$OSiR^6{}_3$, -OR^6 and -$NR^6{}_2$.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, –NO_2, -R^3, -OR^3, -$SiR^3{}_3$

R^9 is =O, -F, -Cl, -Br, -I, -CN, -NO_2, -OR^6, -$NR^6{}_2$, -NR^6-$C(O)R^8$, -NR^6-$C(O)OR^8$, -SR^6, -$S(O)R^6$, -$S(O)_2R^6$, -$COOR^6$, -$C(O)NR^6{}_2$ and -$S(O)_2NR^6{}_2$.

Cross-link cleavage building blocks

It may be advantageous to split the transfer of a chemical entity to a recipient reactive group into two separate steps, namely a cross-linking step and a cleavage step because each step can be optimized. A suitable building block for this two step process is illustrated below:



Initally, a reactive group appearing on the functional entity precursor (abbreviated FEP) reacts with a recipient reactive group, e.g. a reactive group appearing on a scaffold, thereby forming a cross-link. Subsequently, a cleavage is performed, usually by adding an aqueous oxidising agent such as I_2, Br_2, Cl_2, H^+, or a Lewis acid. The cleavage results in a transfer of the group HZ-FEP- to the recipient moiety, such as a scaffold.

In the above formula

Z is O, S, NR^4

Q is N, CR^1

P is a valence bond, O, S, NR^4, or a group C_{5-7}arylene, C_{1-6}alkylene, C_{1-6}O-alkylene, C_{1-6}S-alkylene, NR^1-alkylene, C_{1-6}alkylene-O, C_{1-6}alkylene-S option said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

B is a group comprising D-E-F, in which

D is a valence bond or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

E is, when present, a valence bond, O, S, NR^4, or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

F is, when present, a valence bond, O, S, or NR^4,

A is a spacing group distancing the chemical structure from the complementing element, which may be a nucleic acid,

R^1, R^2, and R^3 are independent of each other selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-$NR^4{}_2$, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-$NR^4{}_2$, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

FEP is a group selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-$NR^4{}_2$, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-$NR^4{}_2$, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, -CNO, $-C(NOH)NH_2$, -NHOH, -NHNHR^6, $-C(O)R^6$, $-SnR^6{}_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and R^7 is independently selected from $–NO_2$, $-COOR^6$, $-COR^6$, -CN, $-OSiR^6{}_3$, $-OR^6$ and $-NR^6{}_2$.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, $–NO_2$, $-R^3$, $-OR^3$, $-SiR^3{}_3$

R^9 is =O, -F, -Cl, -Br, -I, -CN, $-NO_2$, $-OR^6$, $-NR^6{}_2$, $-NR^6$-$C(O)R^8$, $-NR^6$-$C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6{}_2$ and $-S(O)_2NR^6{}_2$.

In a preferred embodiment Z is O or S, P is a valence bond, Q is CH, B is CH_2, and R^1, R^2, and R^3 is H. The bond between the carbonyl group and Z is cleavable with aqueous I_2.

Contacting between target and library

The contacting step, by which the library of bifunctional molecules is subjected under binding conditions to a target associated with a target oligonucleotide, may be referred to as the enrichment step or the selection step, as appropriate, and includes the screening of the library for display molecules having predetermined desirable characteristics. Predetermined desirable characteristics can include binding to a target, catalytically changing the target, chemically reacting with a target in a manner which alters/modifies the target or the functional activity of the target, and covalently attaching to the target as in a suicide inhibitor.

In theory, display molecules of interest can be selected based on their properties using either physical or physiological procedures. The method preferred according to the present invention is to enrich molecules with respect to binding affinity towards a target of interest. In a certain embodiment, the basic steps involve mixing the library of complexes with the immobilized target of interest. The target can be attached to a column matrix or microtitre wells with direct immobilization or by means of antibody binding or other high-affinity interactions. In another embodiment, the target and displayed molecules interact without immobilisation of the target. Displayed molecules that bind to the target will be retained on this surface, while nonbinding displayed molecules in a certain aspect of the invention will be removed during a single or a series of wash steps. The identifier oligonucleotides of complexes bound to the target can then be coupled to the target oligonucleotide. It may be considered advantageously to perform a chromatography step after or instead of the washing step, notably in cases where the target is not immobilized. After the coupling between the identifier oligonucleotide and the

target oligonucleotide, the coupled oligonucleotide may be recovered and optionally amplified before the decoding step.

A significant reduction in background binders may be obtained with increased washing volumes, repeating washing steps, higher detergent concentrations and prolonged incubation during washing. Thus, the more volume and number of steps used in the washing procedure together with more stringent conditions the more efficiently the non-binders and background binders will be removed. The right stringency in the washing step can also be used to remove low-affinity specific binders. However, the washing step will also remove wanted binders if too harsh conditions are used.

A blocking step, such as incubation of solid phase with skimmed milk proteins or other inert proteins and/or mild detergent such as Tween-20 and Triton X-100, may also be used to reduce the background. The washing conditions should be as stringent as possible to remove background binding but to retain specific binders that interact with the immobilized target. Generally, washing conditions are adjusted to maintain the desired affinity binders, e.g. binders in the micromolar, nanomolar, or pocomolar range.

In traditional elution protocols, false positives due to suboptimal binding and washing conditions are difficult to circumvent and may require elaborate adjustments of experimental conditions. However, an enrichment of more than 100 to 1000 is rarely obtained. The present invention alleviates the problem with false positive being obtained because the non-specific binding complexes to a large extent remain in solution or attached to the reaction chamber such that the indentifier oligonucleotide of non-binding complexes will be in a low concentration compared to the identifier oligonucleotides of binding complexes relative to the concentration of the target oligonucleotide.

The target can be any compound of interest. E.g. the target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, an-

tigen, antibody, virus, substrate, metabolite, transition state analogue, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc. without limitation. Suitable targets include, but are not limited to, angiotensin converting enzyme, renin, cyclooxygenase, 5-lipoxygenase, IIL- 1 0 converting enzyme, cytokine receptors, PDGF receptor, type II inosine monophosphate dehydrogenase, β-lactamases, integrin, proteases like factor VIIa, kinases like Bcr-Abl/Her, phosphotases like PTP-1B, and fungal cytochrome P-450. Targets can include, but are not limited to, bradykinin, neutrophil elastase, the HIV proteins, including *tat, rev, gag, int,* RT, nucleocapsid etc., VEGF, bFGF, TGFβ, KGF, PDGF, GPCR, thrombin, substance P, IgE, sPLA2, red blood cells, glioblastomas, fibrin clots, PBMCs, hCG, lectins, selectins, cytokines, ICP4, complement proteins, etc.

Certain targets comprise one or more discrete binding domains. Proteins that contain these domains are involved in a variety of processes, such as cellular transporters, cholesterol movement, signal transduction and signaling functions which are involved in development and neurotransmission. See *Herz, Lipoprotein receptors: beacons to neurons?*, (2001) Trends in Neurosciences 24(4):193-195; Goldstein and Brown,The Cholesterol Quartet, (2001) Science 292:1310-1312. The function of a discrete binding domain is often specific but it also contributes to the overall activity of the protein or polypeptide. For example, the LDL-receptor class A domain (also referred to as a class A module, a complement type repeat or an A-domain) is involved in ligand binding while the gamma-carboxyglumatic acid (Gla) domain which is found in the vitamin-K-dependent blood coagulation proteins is involved in high-affinity binding to phospholipid membranes. Other discrete binding domains include, e.g., the epidermal growth factor (EGF)-like domain in tissue-type plasminogen activator which mediates binding to liver cells and thereby regulates the clearance of this fibrinolytic enzyme from the circulation and the cytoplasmic tail of the LDL-receptor which is involved in receptormediated endocytosis.

Individual target proteins can possess one or more discrete monomer domains, as discussed above. These proteins are often called mosaic proteins. For example, members of the LDL-receptor family contain four major structural domains: the cysteine rich A-domain repeats, epidermal growth factor precursor-like repeats, a transmembrane domain and a cytoplasmic domain. The LDL-receptor family includes members that: 1) are cell-surface receptors; 2) recognize extracellular ligands; and 3) internalize them for degradation by lysosomes. See Hussain et al., *The Mammalian Low-Density Lipoprotein Receptor Family*, (1999) Annu. Rev. Nutr. 19:141-72. For example, some members include very-low-density lipoprotein receptors (VLDL-R), apolipoprotein E receptor 2, LDLR-related protein (LRP) and megalin. Family members have the following characteristics: 1) cell-surface expression; 2) extracellular ligand binding consisting of A-domain repeats; 3) requirement of calcium for ligand binding; 4) recognition of receptor-associated protein and apolipoprotein (apo) E; 5), epidermal growth factor (EGF) precursor homology domain containing YWTD repeats; 6) single membrane-spanning region; and 7) receptor-mediated endocytosis of various ligands. See Hussain, *supra*. Yet, the members bind several structurally dissimilar ligands. The present invention offers the possibility of identifying two or more ligands against the same target as discussed elsewhere herein.

In some aspects of the invention, a dimer compound binding with two targets normally interacting in a biological context is identified using the methods of the present invention. Examples of such targets are factor Xa and factor VIIa. The method includes separately screening a library of bifunctional complexes and identifying suitable display molecules that binds to both targets. The two display molecules pools are then linked together. In the event m display molecules are identified having an affinity above a certain threshold (i.e. a low Kd) to a first target and n display molecules having an affinity above another or the same threshold, a dimer compound array of n times m molecules are formed. The dimer compound are subsequently screened for the ability to bind to the first and the second target molecule, thereby identifying a dimer or

a range of dimers that specifically bind to the first and the second target molecule with a certain affinity.

A target can also be a surface of a non-biological origin, such as a polymer surface or a metal surface. The method of the invention may then be used to identify suitable coatings for such surfaces.

In a preferred embodiment, the desirable display molecule acts on the target without any interaction between the nucleic acid attached to the desirable encoded molecule and the target. In one embodiment, the bound complex-target aggregate can be partitioned from unbound complexes prior to or subsequent to the coupling step by a number of methods. The methods include nitrocellulose filter binding, column chromatography, filtration, affinity chromatography, centrifugation, and other well known methods. A preferred method is size-exclusion chromatography.

Briefly, the library of complexes is subjected to the target, which may include contact between the library and a column onto which the target is immobilised. Identifier oligonucleotides associated with undesirable display molecules, i.e. display molecules not bound to the target under the stringency conditions used, will pass through the column. Additional undesirable display molecules (e.g. display molecules which cross-react with other targets) may be removed by counter-selection methods. Desirable complexes are bound to the column. The target may be immobilized in a number of ways. In one embodiment, the target is immobilized through a cleavable physical link, such as one more chemical bonds. Following the interaction of the display molecule and the target, the respective oligonucleotides are coupled. The aggregate of the target and the complex may then be subjected to a size exclusion chromatography to separate the aggregate from the rest of the compounds in the media.

The complex may be provided with a cleavable linker at a position between the display molecule and the identifier oligonucleotide. When the target is immobilized the cleavable linker of the complex is preferable orthogonal to the cleavable linker that attached the target to the solid support. Subsequent to the optional size exclusion chromatography, this cleavable linker is cleaved to separate the identifier oligonucleotides of complexes having affinity towards the targets. Just to mention a single type of orthogonal cleavable linkages, one could attached to target to the solid support through a linkage that can be cleaved by a chemical agent, and the linker separating the display molecule and the identifier oligonucleotide may be selected as a photocleavable linkage. More specifically, the former linkage may be a disulphide bond that can be cleaved by a suitable reducing agent like DTT (dithiothreitol) and the latter linkage may be an *o*-nitrophenyl group.

There are other partitioning and screening processes which are compatible with this invention that are known to one of ordinary skill in the art. Such known process may be used in combination with the present inventive method. In one embodiment, the coupling products can be fractionated by a number of common methods and then each fraction is assayed for activity. The fractionization methods can include size, pH, hydrophobicity, etc.

Inherent in the present method is the selection of encoded molecules on the basis of a desired function; this can be extended to the selection of molecules with a desired function and specificity. Specificity can be required during the selection process by first extracting complexes which are capable of interacting with a non-desired "target" (negative selection, or counter-selection), followed by positive selection with the desired target. As an example, inhibitors of fungal cytochrome P-450 are known to cross-react to some extent with mammalian cytochrome P-450 (resulting in serious side effects). Highly specific inhibitors of the fungal cytochrome could be selected from a library by first removing those complexes capable of interacting with the

mammalian cytochrome, followed by retention of the remaining products which are capable of interacting with the fungal cytochrome.

The present invention can be used to identify compounds that bind to different molecular targets. A small library of preferred compounds can be directly linked to an oligonucleotide that identifies the structure of the said compound. This can be done manually or using a more automatic system such as robotic equipments. These tagged compounds can then be mixed with one or more target molecules to select for compound and target pairs that bind to each other. For example, a library of compounds designed to bind preferably to protein kinases could be mixed with a library of various protein kinases to identify compounds that specifically interact with a certain protein kinase. The use of the present proximity selection procedure will in this instance generate an extensive structure activity relationship (SAR) where different binding compound are match simultaneously against different related target molecules. The above method can also be used for other target classes such as proteases, phosphatases, GPCRs, nuclear receptors and corresponding compound libraries. The information for these selections can be used to study the selectivity and specificity and to design sub-libraries with potential binding compounds.

In a prior art selection where the target is immobilized to a surface, for example in a well or a bead, the effective concentration of the target will be high locally on the surface but infinitively low in the solution. This restriction will result in low recovery of binding molecules because most of the binding molecules are free in the solution and removed in future washing steps. The present invention allows selection in solution at true equilibrium conditions between the molecular target and the binding molecules. The invention is not dependent on washing- or separation-step as is most prior art selection protocols. The amount of captured binding molecules can be varied using a suitable concentration of the target. Most of the binding molecules with a certain binding constant can be captured if the target concentration is higher than the

binding constant of the binding molecules. Also the concentration of the target can be adjusted to capture binders with a certain binding constant. A high target concentration will also increase the likelihood of selecting specific binders that are present in low copy number. By using high concentration of target, the solution selection can also be used to identify binders with low binding affinity. This is especially important with screening for small fragments which normally possess low affinity but still holds important structural information that can be used in second generation library design.

In a certain embodiment, a binding platform may be constructed that can be used for almost any target. The binding platform should preferably be small enough to only allow association of a few or a single target molecule. This to ensure a solution based selection procedure with adjustable target concentration. The binding platform is primarily composed of two components; a small surface allowing association of the target molecule, and an association area/site for the target oligonucleotide. This binding platform may be designed to mediate the association of the target and target oligonucleotide to allow proximity selection in solution.

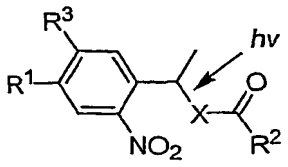
Cleavable linkers

A cleavable linker may be positioned between the target and a solid support, between the potential drug candidate and the identifier oligonucleotide, between the molecular target and the target oligonucleotide or any other position that can provide for a separation of the identifier oligonucleotide from successful complexes from non-specific binding complexes. The cleavable linker may be selectively cleavable, i.e. conditions may selected that only cleave that particular linker.

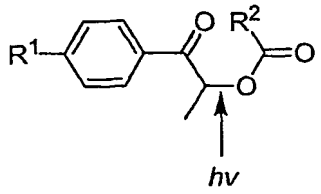
The cleavable linkers may be selected from a large plethora of chemical structures. Examples of linkers includes, but are not limited to, linkers having an enzymatic cleavage site, linkers comprising a chemical degradable component, and linkers cleavable by electromagnetic radiation, such as light.

Examples of linkers cleavable by electromagnetic radiation (light)

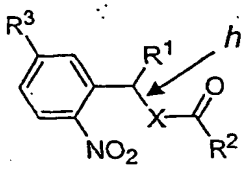
o-nitrobenzyl



p-alkoxy

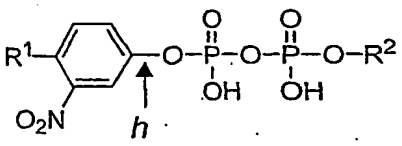


o-nitrobenzyl in exo position



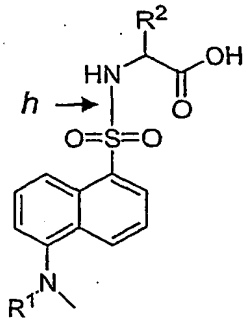
For more details see Holmes CP. J. Org. Chem. **1997,** 62, 2370-2380

3-nitrophenyloxy



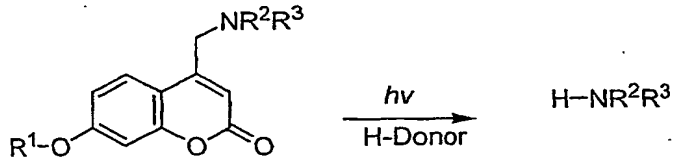
For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

Dansyl derivatives:



For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

Coumarin derivatives



For more details see R. O. Schoenleber, B. Giese. Synlett **2003**, 501-504

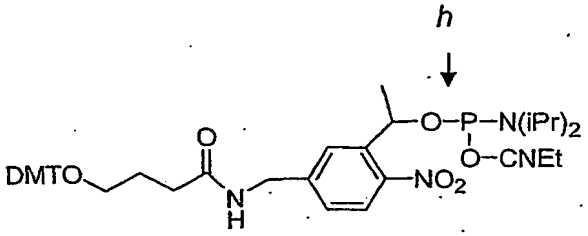
R^1 and R^2 can be either of the potential drug candidate and the identifier oligonucleotide, respectively. Alternatively, R^1 and R^2 can be either of the target or a solid support, respectively.

R^3 = H or OCH_3

If X is O then the product will be a carboxylic acid

If X is NH the product will be a carboxamide

One specific example is the PC Spacer Phosphoramidite (Glen research catalog # 10-4913-90) which can be introduced in an oligonucleotide during synthesis and cleaved by subjecting the sample in water to UV light (~ 300-350 nm) for 30 seconds to 1 minute.

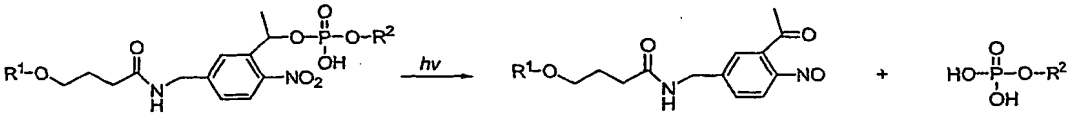


DMT = 4,4'-Dimethoxytrityl

iPr = Isopropyl

CNEt = Cyanoethyl

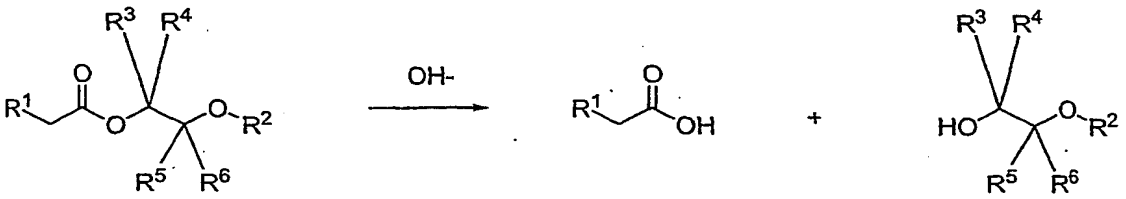
The above PC spacer phosphoamidite is suitable incorporated in a library of complexes at a position between the indentifier and the potential drug candidate. The spacer may be cleaved according to the following reaction.



R^1 and R^2 can be either of the encoded molecule and the identifying molecule, respectively. In a preferred aspect R^2 is an oligonucleotide identifier and the R^1 is the potential drug candidate. When the linker is cleaved a phosphate group is generated allowing for further biological reactions. As an example, the phosphate group may be positioned in the 5´end of an oligonucleotide allowing for an enzymatic ligation process to take place.

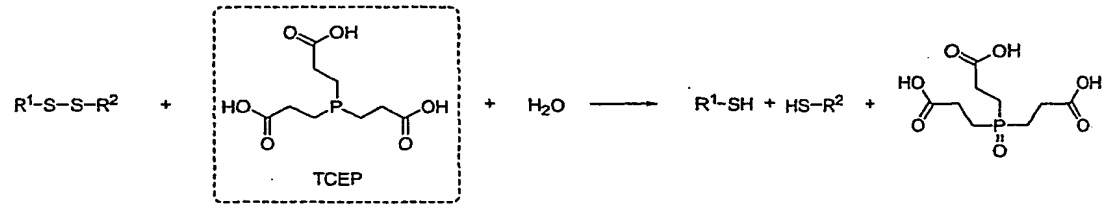
Examples of linkers cleavable by chemical agents:

Ester linkers can be cleaved by nucleophilic attack using e.g. hydroxide ions. In practice this can be accomplished by subjecting the target-ligand complex to a base for a short period.



R^1 and R^2 can be the either of be the potential drug candidate or the identifier oligonucleotide, respectively. R^{4-6} can be any of the following: H, CN, F, NO_2, SO_2NR_2.

Disulfide linkers can efficiently be cleaved / reduced by Tris (2-carboxyethyl) phosphine (TCEP). TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range. These reductions frequently required less than 5 minutes at room temperature. TCEP is a non-volatile and odorless reductant and unlike most other reducing agents, it is resistant to air oxidation. Trialkylphosphines such as TCEP are stable in aqueous solution, selectively reduce disulfide bonds, and are essentially unreactive toward other functional groups commonly found in proteins.



More details on the reduction of disulfide bonds can be found in Kirley, T.L.(1989), Reduction and fluorescent labeling of cyst(e)ine-containing pro-

teins for subsequent structural analysis, *Anal. Biochem.* **180**, 231 and Levison, M.E., *et al.* (1969), Reduction of biological substances by water-soluble phosphines: Gamma-globulin. *Experentia* **25**, 126-127.

Linkers cleavable by enzymes

The linker connecting the potential drug candidate with the identifier oligonucleotide or the solid support and the target can include a peptide region that allows a specific cleavage using a protease. This is a well-known strategy in molecular biology. Site-specific proteases and their cognate target amino acid sequences are often used to remove the fusion protein tags that facilitate enhanced expression, solubility, secretion or purification of the fusion protein.

Various proteases can be used to accomplish a specific cleavage. The specificity is especially important when the cleavage site is presented together with other sequences such as for example the fusion proteins. Various conditions have been optimized in order to enhance the cleavage efficiency and control the specificity. These conditions are available and know in the art.

Enterokinase is one example of an enzyme (serine protease) that cut a specific amino acid sequence. Enterokinase recognition site is Asp-Asp-Asp-Asp-Lys (DDDDK), and it cleaves C-terminally of Lys. Purified recombinant Enterokinase is commercially available and is highly active over wide ranges in pH (pH 4.5-9.5) and temperature (4-45°C).

The nuclear inclusion protease from tobacco etch virus (TEV) is another commercially available and well-characterized proteases that can be used to cut at a specific amino acid sequence. TEV protease cleaves the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly/Ser (ENLYFQG/S) between Gln-Gly or Gln-Ser with high specificity.

Another well-known protease is thrombin that specifically cleaves the sequence Leu-Val-Pro-Arg-Gly-Ser (LVPAGS) between Arg-Gly. Thrombin has

also been used for cleavage of recombinant fusion proteins. Other sequences can also be used for thrombin cleavage; these sequences are more or less specific and more or less efficiently cleaved by thrombin. Thrombin is a highly active protease and various reaction conditions are known to the public.

Activated coagulation factor FX (FXa) is also known to be a specific and useful protease. This enzyme cleaves C-terminal of Arg at the sequence Ile-Glu-Gly-Arg (IEGR). FXa is frequently used to cut between fusion proteins when producing proteins with recombinant technology. Other recognition sequences can also be used for FXa.

Other types of proteolytic enzymes can also be used that recognize specific amino acid sequences. In addition, proteolytic enzymes that cleave amino acid sequences in an un-specific manner can also be used if only the linker contains an amino acid sequence in the complex molecule.

Other type of molecules such as ribozymes, catalytically active antibodies, or lipases can also be used. The only prerequisite is that the catalytically active molecule can cleave the specific structure used as the linker, or as a part of the linker, that connects the encoding region and the displayed molecule or, in the alternative the solid support and the target.

A variety of endonucleases are available that recognize and cleave a double stranded nucleic acid having a specific sequence of nucleotides. The endonuclease Eco RI is an example of a nuclease that efficiently cuts a nucleotide sequence linker comprising the sequence GAATTC also when this sequence is close to the nucleotide sequence length. Purified recombinant Eco RI is commercially available and is highly active in a range of buffer conditions. As an example the Eco RI is working in in various protocols as indicted below (NEBuffer is available from New England Biolabs):

NEBuffer 1 : [10 mM Bis Tris Propane-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.0 at 25°C)],

NEBuffer 2 : [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.9 at 25°C)],

NEBuffer 3 : [100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.9 at 25°C)],

NEBuffer 4 : [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 at 25°C)].

Extension buffer : mM KCl, 20 mM Tris-HCl(Ph 8.8 at 25o C), 10 mM (NH4)2 SO4 , 2 mM MgSO 4 and 0.1% Triton X-100, and 200 µM dNTPs.

Determining the identifier oligonucleotide sequence

The nucleotide sequence of the identifier sequence present in the coupled product is determined to identify the identity of the binding display molecule(s) and7or optionally the molecular target(s). In a certain embodiment of the invention, chemical entities that participated in the formation of the display molecules that binds to the target are identified. The synthesis method of the display molecule may be established if information on the chemical entities as well as the point in time they have been incorporated in the display molecule can be deduced from the identifier oligonucleotide. It may be sufficient to obtain information on the chemical structure of the various chemical entities that have participated in the display molecule to deduce the full molecule due to structural constraints during the formation. As an example, the use of different kinds of attachment chemistries may ensure that a chemical entity on a building block can only be transferred to a single position on a scaffold. Another kind of chemical constrains may be present due to steric hindrance on the scaffold molecule or the chemical entity to be transferred. In general however, it is preferred that information can be inferred from the identifier oligonucleotide sequence that enable the identification of each of the chemical entities that have participated in the formation of the encoded

molecule along with the point in time in the synthesis history the chemical entities have been incorporated in the (nascent) display molecule.

Although conventional DNA sequencing methods are readily available and useful for this determination, the amount and quality of isolated bifunctional molecule may require additional manipulations prior to a sequencing reaction.

Where the amount is low, it is preferred to increase the amount of the coupled oligonucleotide sequence by polymerase chain reaction (PCR) using PCR primers directed to primer binding sites present in the identifier oligonucleotide sequence.

In one embodiment, the different coupled oligonucleotide sequences are cloned into separate sequencing vectors prior to determining their sequence by DNA sequencing methods. This is typically accomplished by amplifying the different coupled oligonucleotide sequences by PCR and then using a unique restriction endonuclease sites on the amplified product to directionally clone the amplified fragments into sequencing vectors. The cloning and sequencing of the amplified fragments then is a routine procedure that can be carried out by any of a number of molecular biological methods known in the art.

Alternatively, the bifunctional complex or the PCR amplified identifier oligonucleotide sequence can be analysed in a microarray. The array may be designed to analyse the presence of a single codon or multiple codons in a identifier oligonucleotide sequence.

In still another approach, the coupled oligonucleotide product is analysed by QPCR. Preferably, the QPCR affords information as to the chemical moieties that has participated in the formation of the display molecules and optionally the identity of the target. The QPCR approach also allows a direct investiga-

tion of the enrichment factor if two samples are analysed in parallel, one with target and the other with the target plus library. The difference in signal from these to samples will illustrate how much coupling that is due to the target mediated coupling compared to the background coupling. Various conditions can be investigated to obtain the most optimal selection procedure before the sequences are analysed to identify the precise structures of the binding molecules.

Synthesis of nucleic acids

Oligonucleotides can be synthesized by a variety of chemistries as is well known in the art. For synthesis of an oligonucleotide on a substrate in the direction of 3' to 5', a free hydroxy terminus is required that can be conveniently blocked and deblocked as needed. A preferred hydroxy terminus blocking group is a dimexothytrityl ether (DMT). DMT blocked termini are first deblocked, such as by treatment with 3% dichloroacetic acid in dichloromethane (DCM) as is well known for oligonucleotide synthesis, to form a free hydroxy terminus.

Nucleotides in precursor form for addition to a free hydroxy terminus in the direction of 3' to 5' require a phosphoramidate moiety having an aminodiisopropyl side chain at the 3' terminus of a nucleotide. In addition, the free hydroxy of the phosphoramidate is blocked with a cyanoethyl ester (OCNET), and the 5' terminus is blocked with a DMT ether. The addition of a 5' DMT-, 3' OCNET-blocked phosphoramidate nucleotide to a free hydroxyl requires tetrazole in acetonitrile followed by iodine oxidation and capping of unreacted hydroxyls with acetic anhydride, as is well known for oligonucleotide synthesis. The resulting product contains an added nucleotide residue with a DMT blocked 5' terminus, ready for deblocking and addition of a subsequent blocked nucleotide as before.

For synthesis of an oligonucleotide in the direction of 5' to 3', a free hydroxy terminus on the linker is required as before. However, the blocked nucleotide

to be added has the blocking chemistries reversed on its 5' and 3' termini to facilitate addition in the opposite orientation. A nucleotide with a free 3' hydroxyl and 5' DMT ether is first blocked at the 3' hydroxy terminus by reaction with TBS-Cl in imidazole to form a TBS ester at the 3' terminus. Then the DMT-blocked 5' terminus is deblocked with DCA in DCM as before to form a free 5' hydroxy terminus. The reagent (N,N-diisopropylamino)(cyanoethyl) phosphonamidic chloride having an aminodiisopropyl group and an OCNET ester is reacted in tetrahydrofuran (THF) with the 5' deblocked nucleotide to form the aminodiisopropyl-, OCNET-blocked phosphonamidate group on the 5' terminus. Thereafter the 3' TBS ester is removed with tetrabutylammonium fluoride (TBAF) in DCM to form a nucleotide with the phosphonamidate-blocked 5' terminus and a free 3' hydroxy terminus. Reaction in base with DMT-Cl adds a DMT ether blocking group to the 3' hydroxy terminus.

The addition of the 3' DMT-, 5' OCNET-blocked phosphonamidated nucleotide to a linker substrate having a free hydroxy terminus then proceeds using the previous tetrazole reaction, as is well known for oligonucleotide polymerization. The resulting product contains an added nucleotide residue with a DMT-blocked 3' terminus, ready for deblocking with DCA in DCM and the addition of a subsequent blocked nucleotide as before.

Brief Description of the Figures

Fig. 1 discloses an embodiment for proximity-dependent selection,

Fig. 2 discloses different approaches for accomplishing coupling,

Fig. 3 discloses four different approaches for producing a coupling

Fig. 4 discloses a library versus library screening,

Fig. 5 discloses an embodiment in which a target oligonucleotide association is performed in solution.

Fig. 6 discloses a target oligonucleotide association on cell surface.

Fig. 7 discloses a target with multiple binding sites which may associate with members of the bifunctional molecule library.

Fig. 8 discloses a target with one binding site for association with a pair of displayed molecules.

Fig. 9 discloses a 2nd generation-library driven proximity selection.

Fig. 10 discloses multiple targets for simultaneously subjected to a library of complexes.

Detailed Disclosure of the Figures

Fig. 1 outlines an embodiment for a proximity-dependent selection (PDS). The molecular target is linked to a target oligonucleotide, which in some embodiment may be unique for the target molecule. This target sequence comes in close proximity with a specific identifier oligonucleotide when the displayed molecule of a bifunctional complex binds to the target molecule. This proximity will promote the coupling between the bifunctional complex molecules that bind to the target compare to bifunctional complex in solution. Thus, there will be a selection for coupling products that contain display molecules that possess affinity for the target molecule. The final ligation product is amplified using two primers that only amplify ligated products.

In a first step, the target associate with a target oligonucleotide is mixed with a library of complexes, in which each complex comprises a display molecule attached to an identifier oligonucleotide. The display molecules are then incubated with the target. The display molecules which have an affinity towards the molecular target will bind, while the complexes not having affinity will remain in solution. Subsequent to the incubation, a connector oligonucleotide is added. The connector oligo nucleotide comprises parts that hybridise to sequences near the ends of the target and the identifier oligonucleotides, respectively. Subsequent to the addition of the connector oligonucleotide, a ligation is effected by chemical or enzymatic means. Preferably a ligase is used to ligate the target and the identifier oligonucleotides together. The connector oligonucleotide is generally added in excess to saturate the complexes in solution to avoid unspecific ligation.

After the ligation, the ligation product is amplified by PCR. Thus, a forward primer is annealed to the ligation product at the 3' end thereof and extended using a polymerase. The transcribed product comprises a site to which a second (or reverse) primer can anneal so as to provide for an extension of the second primer using the transcribed product as template. Using forward and reverse primers as indicated above together with a polymerase and suitable substrates produces amplicons, which comprises information about the display molecule as well as the molecular target. The ligated product can be introduced into a host organism using a suitable vector. The host vector may be allowed to form colonies and the colonies can be sequenced to establish the identity of the display molecule.

Fig. 2 shows various options to perform coupling between the target oligonucleotide and the identifier oligonucleotide. **A.** The ligation is promoted using a connector oligonucleotide that anneals both to the target oligonucleotide and the identifier oligonucleotide. The connector oligonucleotide is designed such that the ends of the identifier oligonucleotide and the target oligonucleotides are abutted. A ligase is subsequently allowed to ligate the ends together. **B.** A connector oligonucleotide is used to promote fill in of a gap using a polymerase and finally ligation using a ligase. **C.** The distal end of the target oligonucleotide overlaps the distal end of the identifier oligonucleotide, which allows a polymerase to extend the target oligonucleotide as well as the identifier oligonucleotide thereby forming a double stranded product. **D.** Blunt-ended ligation of single-stranded or double stranded DNA using a suitable enzyme like T4 DNA ligase.

Fig. 3 shows various methods for preparing a coupling area on an existing bifunctional complex. Conjugates between molecular targets associated with an oligonucleotide and complexes comprising a display molecule and an identifier oligonucleotide can be modified to allow a ligase to couple the oligonucleotides together. **A.** The identifier oligonucleotide is extended with a primer with an overhang that creates the coupling area. The extension is

suitably conducted before the selection process to obtain the benefit of a double stranded nucleotide sequence. A target oligonucleotide can be ligated to the blunt end of the extended primer or a connector oligonucleotide can be used to connect the target oligonucleotide and the extended primer prior to ligation with a suitable ligase. **B.** The identifier oligonucleotide is annealed to a primer that binds internally. The primer is subsequently extended, suitably before the selection process. The extension forms a coupling area directly on the identifier oligonucleotide, which allows a target oligonucleotide to be annealed and ligated. **C.** The first step is identical to the procedure as describe in B but the target sequence has a free 5'-end that allow ligation to the 3'-end of the identifier oligonucleotide. A blunt ended single stranded ligation can be performed. Alternatively, this variation can be performed using a connector oligonucleotide and subsequent ligation. **D.** A primer is annealed to a identifier oligonucleotide and extended to produce a double-stranded DNA which is subsequently cut with an enzyme (*e.g.* restriction enzyme) to produce a single-stranded DNA portion that can be used as handle in the coupling process.

Fig. 4 shows a library versus library selection method. Different targets specifically encoded by the attached target oligonucleotides are mixed with a library of bifunctional complexes. The displayed molecules will bind to specific targets and promote the ligation through the proximity effect. This ligation will connect the target oligonucleotides with oligonucleotides that encodes for specific displayed molecules. The ligated oligonucleotides can be amplified and determined by sequencing procedures well known in the art. The ligated sequences will reveal which display molecules that bind to which target.

Fig. 5 discloses *inter alia* the association of the target oligonucleotide to the target. One way of associating the target oligonucleotide with the target molecule is to link the oligonucleotide through a tag introduced on the target molecule. The tag can be attached before the target is produced (*e.g.* a short

amino acid sequence such as HIS-tag of FLAG-tag) or be modified after the target is produced. The target sequence can then be associated through the tag using a tag-binding molecule such as an antibody or other type of molecules that binds to the tag.

Fig. 6 discloses target oligonucleotide association on a cell surface. Specific receptors can be engineered to express a specific tag on the cell surface. Different tags can be used such as HIS- or FLAG-tags or other types of tags that become bound with the receptor. The tag will only be displayed on the cell surface together with the specific receptor. The target oligonucleotide is then associated with the receptor target using a mediator molecule that carries the target oligonucleotide and binds to the tag. A mediator molecule could be an antibody that binds to the tag (*e.g.* anti-HIS or anti-FLAG antibodies) that is associated with the target oligonucleotide. This procedure will specifically associate the target oligonucleotide with a receptor target on the cell surface which will promote a ligation between oligonucleotides of the binding displayed molecules and the target oligonucleotide.

Fig. 7 shows a target molecule with several sites for binding of ligands. The target is subjected to a library of complexes of bifunctional molecules. Display molecules of the complexes binds to the discrete sites of the molecular target thus promoting a high local concentration of the ends of the oligonucleotides which have bound to the target. Subsequently a connector oligonucleotide is added to adjoin the distal ends of the oligonucleotides together. Usually, the connector oligonucleotide is added in excess to saturate the ends of the identifier oligonucleotides free in the solution. The ends of the oligonucleotides kept together by the connector oligonucleotide are ligated together forming a coupled product. The coupled product is amplified by PCR using primers annealing to each end of the coupled product. The amplified coupled product is decoded to identify the display molecules which have bound to the in the target. In a step not shown on the figure, the two binding display molecules are coupled together via a suitable linker to form a ligand

which binds to two sites of the target. Suitable, the dimer comprising the two revealed display molecules and the linker is synthesised by organic synthesis.

Libraries of bifunctional complexes can also be screened against each other using the present invention. Such an embodiment allows the detecting of pairs of displayed molecules that bind to the same target at different or the same binding site or pair of displayed molecules that bind to different targets. The power of the screening libraries in the above fashion is indicated by the fact that a library of e.g. 10^4 different displayed molecules generates a total combination of display molecules of 10^8 when pair of binders are searched for.

Fig. 8 discloses a library of bifunctional complexes which is presented to a target having a site possible to be occupied by two display molecules. Initially, the target is mixed with the library of bifunctional complexes under conditions which promote a binding interaction to take place. A first bifunctional complex associates with the target to form the target associated with the target oligonucleotide. Subsequently to or simultaneously with the binding of the first display molecule, a second complex binds to the same site of the target. The display molecules may or may not be reacted with each other to form a covalent linkage between the display molecules. In another embodiment, the two display molecules are connected *via* a suitable linker or reacted with an external reactant so as to form a single molecule. After the binding interaction of the library of complexes with the target, the ends of the complexes which comprises display molecules that binds to the target are joint together. In an aspect of the invention, the ends are joined together using a connector polynucleotide. The connector polynucleotide is preferably added in excess to saturate ends of identifier oligonucleotides which are not part of a binding complex. After the hybridisation event between the ends of the identifier oligonucleotides and the connector oligonucleotide a ligation is conducted. Suitably the ligation is performed by a ligase to form a coupled product,

which can be used as a template by a polymerase. After the ligation, the coupled product is amplified by PCR to form PCR amplicons comprising information of the display molecules which have participated in the binding interaction.

Fig. 9 discloses a two (or more) step identification method. In a first step the method as disclosed in figure 1 is conducted and in the second step a new library prepared upon the knowledge harvested in the first library is used to generate the second generation library. Initially, a library of complexes are is presented to a target having a binding site. In the library, display molecules having a binding affinity above a certain threshold is not present, illustrated on the drawing with a display molecule only having a partial fit in the binding site of the target. In the synthesis of the second generation library components used in the synthesis of the low binding display molecule are shuffled with further components and/or the low binding display molecule is added or subtracted a structural unit. As an example, a further round of addition of chemical entities can be conducted. For systems for complex generation relying on the natural translation system a deletion, alteration or addition of nucleic acid can be performed. The second generation library is presented to the a target again. In the event the alteration of the initial low binding molecule has been successful display molecules are generated which binds with a higher affinity towards the target.

Fig. 10 discloses two targets which are attached to each other prior to the mixing with the library of complexes. The attachment can be natural, i.e. the association between target 1 and target 2 occur in a biological context or the attachment can be artificial, i.e. the association between target 1 and target 2 is obtained by a chemical synthesis. In the latter instance, the association between the targets may be obtained by any chemical or enzymatic means which ensure a linkage. The association of the targets may also be obtained by expressing target 1 and target 2 as a fusion protein, i.e. a single protein having two distinct targets or monomer domains. In an embodiment, one of

the targets in the fusion protein is a capturing protein, like streptavidin. In the event the library is spiked with complexes having a ligand against the capturing protein, like biotin, it is feasible to form a connection between the fusion protein and a member of the library. The further functionality, i.e. target 2, of the fusion protein may be then be subjected to a screening process to find binder from the library.

During the mixing step, the two attached targets are contacted with the library of complexes under binding conditions. The library may be spiked with a complex comprising a compounds known to bind to the one of the targets in order to find suitable binders against another target. If the library comprises suitable binding display molecules, two ends of the binding complexes is positioned in close proximity. The addition of a connector oligonucleotide ensures that the ends are kept close together when a ligase is allowed to perform the action of ligating the ends together. The resulting PCR product comprises genetic information which encodes both the display molecules that have participated in the binding interaction with target 1 and target 2.

EXAMPLES illustrating the second aspect of the present invention

Example 1:

Oligonucleotide sequences

Target Sequence (ES-1)

5'-X-TAGTC GATGT AGCTA GCTAG TGCGC CAATG **CCTTA TCAGC**

Identifier Sequence (IS-1) (extension part)

5'-**GATCG ATGAC** TGACG CCGGT AAATCTACCGTCTAAGCTG-Y-3'

Underlined sequence is reverse primer binding site

Control Identifier Sequence (CIS-1) (extension part)

5'-**GATCG ATGAC** TGACG CCGGT *gacgt cgtag atatc gatgc*
AAATCTACCGTCTAAGCTG-Z-3'

Underlined sequence is reverse primer

Connector Sequence (CS-1)

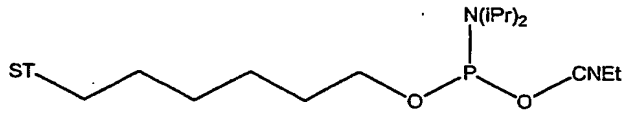
5'-**AAAAGGAATAGTCG-CTAGCTACTGTTTT**

Primers (Forward PR-1 and reverse PR-2)

PR-1: 5'-TAGTC GATGT AGCTA GCTAG
PR-2: 5'-CAGCT TAGAC GGTAG ATTT

Target labelling with oligonucleotide sequence.

The target molecule (streptavidin) is modified with an oligonucleotide sequence using a terminus modifier that allow direct coupling to the target(s) molecule. The oligonucleotide sequence ES-1 is synthesised with the 5'-Thiol-Modifier (Glen Research, #10-1926-90) to produce a oligonucleotide that can be coupled to the target(s) through the 5'-end (designated X in the ES-1).



5'-Thiol-Modifier C6

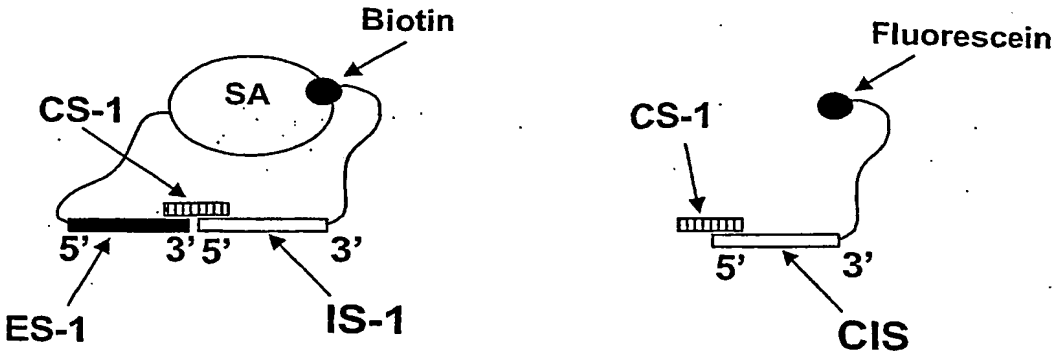
The covalent attachment of the oligonucleotide at the target is carried out with the aid of the heterobispecific crosslinker Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sSMMCC). The ε-amino groups of lysine side chains of the target(s) are first derivatized with sSMCC cross linker to provide a maleimide functionality, which subsequently is reacted with the thiolated oligonucleotide.

Protecol: Dissolve approx. 2 mg of sSMCC in 60 µl of DMF. Add the sSMCC solution to 200 µl of a 100 µM solution of streptavidin in PBS buffer pH 7.3 and incubate in the dark at room temperature for about 1 hour. The excess sSMCC is removed using NAP5 or NAP10 (Pharmacia) using a PBSE buffer. The thiolated oligo (ES-1) is activated in TE buffer, pH 7.4 using 1 mM DDT and excess removed using spin column (BioRad). The activated streptavidin and oligonucleotide are preferably used directly in the cross linking reaction by mixing and incubation for about 1 hour in the dark at room temperature. The modified streptavidin is preferably purified on a size-exclusion column or a anion-exchange column (MonoQ HR5/5, Pharmacia).

Bifunctional complex molecules.

Bifunctional complexes are preferably molecules that are composed of a nucleotide sequence that encodes for the displayed molecule. These complexes can be generated using various procedures, as disclosed elsewhere herein. The bifunctional complexes preferably contain an oligonucleotide that can be ligated or otherwise connected to the oligonucleotide sequence on the target mediated by the binding of the display molecule to the target.

This example describes the bifunctional complex as the IS-1 sequence which is labelled with a biotin in the 3'end (designated Y in the sequence). This oligonucleotide is synthesized using Biotin-dT (Glen Research, # 10-1038-95), which will function as the display molecule and the oligonucleotide sequence encoding the biotin moiety. The displayed biotin molecule has high affinity for streptavidin which will bring the coding oligonucleotide (CS-1) in close proximity of the identifier sequence (IS-1). This will promote the ligation between the ES-1 and the IS-1 oligonucleotides as shown below. The ligation is mediated by the connector sequence (CS-1).



A different bifunctional complex with a Fluorescein as display molecule is also present. The flurorescein will not bind to the target molecule (streptavidin) resulting in no proximity ligation between the ES-1 and the IS-1 oligonucleotides.

Selection through proximity ligation

20 pM bifunctional complex (IS-1) is mixed with 100 pM conjugated target molecule to allow binding of the biotin molecule to streptavidin. Another bifunctional complex (CIS-1) was used as a control (100 pM) with a different display molecule (Fluorescein) included in the synthesis of the oligonucleotide as a Fluorescein-dT (Glen Research, # 10-1056-95). This displayed molecule is encoded by another unique sequence in the CIS-1 oligonucleotide. However, the ligation region is identical to the IS-1 oligonucleotide allowing ligation if proximity is achieved. The CIS-1 oligonucleotide is 10 nucleotides longer permitting the distinction from the IS-1 oligonucleotide by running an agorose gel and determining the length of the oligonucleotides. The mixtures were incubated in 50 mM KCl, 10 mM Tris-HCl. pH 8.3, 1.5 mM $MgCl_2$, 0.15 mM ATP. pH 7.4 for 1 hour to allow association of the Biotin (or Fluorescein) to streptavidin.

The connector sequence (CS-1) is then added at 400 nM concentration to promote the connection between the ES-1 and IS-1 oligonucleotides together with 2 U T4 DNA ligase to start the ligation. The reaction was keep at 30°C

for 5 min and then 80°C for 20 min. The relatively high concentration of the connector oligonucleotide will saturate all IS-1 that have not been brought into proximity of an encoding sequence.

Amplification of the ligated products was performed with the polymerase chain reaction (PCR) using primers corresponding to the 5'-end of the encoding sequence (PR-1: 5'-TAGTC GATGT AGCTA GCTAG) and the 3'-end of the identifier oligonucleotide sequence (PR-2: 5'-CAGCT TAGAC GGTAG ATTT). The primers are design to only amplify the ligated product. PCR was performed using Ready-To-Go (RTG) PCR beads (Amersham Biosciences) and 10 pmol of each primer in a reaction volume of 25 µl. The PCR reaction consisted of an initial denaturation step of 94°C for 2 minutes followed by 20-45 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension step of 2 minutes at 72°C was included. The PCR products were resolved by agarose gel electrophoresis and the band corresponding to the expected size was cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

The different length of the Biotin and Fluorescein identifier oligonucleotide sequences (IS-1 and CIS-1) is used to verify that the bifunctional complex with Biotin have been enriched through the binding to streptavidin.
This example describes a selection using two different bifunctional complexes. The same approach can be used for larger libraries, at least up to 10^{14} different molecules. The same approach is used when screening library versus library. In this case the targets are encoded by different sequences but with identical coupling area.

Cloning/sequencing

To sequence individual PCR fragments the purified PCR products were cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's instructions. The resulting mixture was used for transformation of TOP10 *E. coli* cells (Invitrogen) using standard procedures. The cells were

plated on growth medium containing 100 µg/ml ampicillin and left at 37°C for 12-16 hours. Individual *E.coli* clones were picked and transferred to PCR wells containg 50 µl water. These wells were then boiled for 5 minutes and 20 µl mixture from each well was used in a PCR reaction using RTG PCR beads and 5 pmol each of M13 forward and reverse primers according to the manufacturer's instructions. A sample of each PCR product was then treated with Exonuclease I (USB) and Shrimp Alkaline Phosphatase (USB) to remove degrade single stranded DNA and dNTPs and sequenced using the DYEnamic ET cycle sequencing kit (Amersham Biosciences) according to the manufacturer's instructions and the reactions were analyzed on a MegaBace 4000 capillary sequencer (Amersham Biosciences). Sequence outputs were analyzed with ContigExpress software (Informax Inc.).

Example 2: Library versus library

A library of bifunctional complexes is screened against another library of encoded peptides or proteins. Examples of other encoded peptides are ribosome displayed peptides or mRNA displayed peptides. This example describes the use of bifunctional complexes together with mRNA displayed targets to perform library versus library screening. Any other library, where the genotype is associated with the phenotype, can be used together with bifunctional complexes as described in this invention.

A freshly transcribed mRNA (0.5 – 2.5 nmol) is prepared from an appropriate library. The transcribed mRNA library is hybridized to biotinylated puromycin-linker (about 0.5 nmol) in 300 µl binding buffer (30 mM Tris, pH 7.0, 250 mM NaCl) by heating to 85° C for 30 sec followed by cooling to 4° C in 5 min. 100 µl pre-washed Neutravidin beads (Pierce) is then added to the hybridization mixture and incubated for 30 min at 4 °C under rocking. Subsequently, the beads are washed in 3x 100 µl binding buffer followed by centrifugation to remove the liquid phase. The moist beads were then irradiated for 15 min at room temperature with a 25W UV-lamp (Pyrex-filter, $\lambda > 300$nm). Subsequently, the beads are washed with 100 µl plain water to yield the photo-

crosslinked mRNA-puromycin template which is directly used for mRNA-peptide fusion formation in rabbit reticulocyte lysate to produce the mRNA-protein fusions library according to the literature (Kurz et la., Nucleic Acids Res. 2000, 28:83). The puromycin-linker is also prepared according to Kurz et al. and biotinylated by carbamate bond formation between the puromycin amino group (50 µM) and the photo-cleavable biotin-reagent (NHS-PC-Biotin, 5 mM, EZ-Link ™-Biotin, Pierce Chemicals) in 25% DMSO/water for 2 h at room temperature followed by NaCl/EtOH precipitation.

The mRNA-peptide fusion library is then converted by a suitable primer to contain a coupling area. The coupling area is formed by a primer partly complementary to the distal region of the mRNA and which is able to promote an extension on the mRNA strand with the coupling area. This coupling area is then used in the selection procedure to couple the target oligonucleotide with the identifier oligonucleotide mediated by the binding of displayed molecules to the mRNA-peptide fusions.

1-100 pmol bifunctional complex library molecules are mixed with 1-100 pmol mRNA-peptide fusion library in a binding buffer (50 mM KCl, 10 mM Tris-HCl. pH 8.3, 1.5 mM $MgCl_2$, 0.15 mM ATP. pH 7.4) for 1 hour to allow binding of the displayed molecules to the mRNA-peptide fusion molecules. The connector sequence (an oligonucleotide the is complementary to the coupling area is then added at about 400 nM concentration to promote the connection between the target and identifier oligonucleotides. Subsequently, 2 U T4 DNA ligase is added to start the ligation. The reaction is kept at 30°C for 5 min and then 80°C for 20 min. Amplification of the ligated products is performed using a polymerase chain reaction (PCR) using primers corresponding to the 5'-end of the target oligonucleotide and the 3'-end of the identifier oligonucleotide. The primers are design to only amplify the ligated product. PCR is performed using Ready-To-Go (RTG) PCR beads (Amersham Biosciences) and 10 pmol each primer in a reaction volume of 25 µl. The PCR reaction consisted of an initial denaturation step of 94°C for 2 minutes followed by 20-45

cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension step of 2 minutes at 72°C was included. The PCR products are resolved by agarose gel electrophoresis and the band corresponding to the expected size is cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.